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Pathological findings in a mouse model for Coxsackievirus A16 infection

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Abstract

Coxsackievirus A16 (CV-A16) is the leading cause of hand-foot-mouth disease (HFMD), which usually presents as mild and self-limiting symptoms in young children. Rarely, CV-A16 has been reported to cause severe and fatal neurological complications but little is known about these complications. In the present study, 1-day and 7-day old mouse models of CV-A16 were developed using a clinical strain via subcutaneous inoculation. All infected mice exhibited clinical signs of infection, including reduced mobility, limb weakness and paralysis between 3 to 6 days post-infection. Pathologically, the main organs involved were the central nervous system (CNS), skeletal muscles and brown fat. In the CNS, viral antigens as demonstrated by immunohistochemistry, were localized mainly to neurons in the brain stem and spinal cord, suggesting that CV-A16 is neurotropic although inflammation is very mild. The skeletal muscles showed necrosis and myositis due to viral infection as evidenced by the dense viral antigens. Focal viral antigens were also detected in the brown fat. These preliminary pathological findings indicate that our mouse models can be further developed to be useful models for pathogenesis studies, and vaccine and anti-viral drug evaluation.

INTRODUCTION

Coxsackievirus A16 (CV-A16) is a non-enveloped, single-stranded, positive-sense RNA virus from the family of *Picornaviridae*. First isolated in South Africa in 1951[1], it infects humans via faecal-oral or oral-oral routes.[2,3] Together with enterovirus 71 (EV-A71), CV-A16 is one of the most important pathogens that causes hand-foot-mouth disease (HFMD). The usual mild clinical manifestations of HFMD are fever, vesicles/rashes on the palmar and plantar skin, and ulcers on the buccal mucosa and tongue.[4] Very rarely, severe and fatal neurological complications e.g. rhombencephalitis and myocarditis have been associated with CV-A16.[5-8]

In contrast to EV-A71 encephalomyelitis in which human autopsies have been studied[9-11], human pathology and neuropathogenesis of CV-A16 infection is unknown. Therefore, it is essential to develop animal models to better understand the infectious disease pathology of the virus, particularly its neurotropism. Previously, a few animal models of CV-A16 have been developed as tools for vaccine and anti-viral drug evaluation. In these models, the pathological findings suggest that the virus may have a predilection for the brainstem and cerebellum.[12,13] However, we believe, this has not been convincingly demonstrated. In the present study, a mouse model was established to investigate the neurotropism and viral tropism for non-central nervous system (CNS) tissues of a clinically-isolated CV-A16 strain. The findings in this model extend our knowledge of CV-A16pathogenesis. Moreover, this mouse model could also be potentially useful for testing new anti-viral drugs and vaccines.

METHODS

Virus stock and cell culture

A clinical isolate of CV-A16 (CV-A16-N132), obtained from a HFMD patient was used for our experiments. The virus was propagated in Vero cells maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, USA) maintenance medium supplemented with 2% fetal bovine serum (FBS, JR Scientific, USA). The culture was kept until the cells showed >90% cytopathic effect. After 3 freeze-thaw cycles and centrifugation at 4000rpm for 10 min at 4˚C, the supernatant was filtered through a 0.22μm pore-size filter (Minisart; Sartorius, Germany), aliquoted and stored at -80˚C before use. The viral titers were determined by measuring 50%

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cell culture infective dose ($CCID_{50}$) in a standard microtitration assay in Vero cells as previously described with slight modification.[14] Briefly, monolayers of Vero cells were grown in DMEM growth medium containing 5% FBS in a 96-well plate (Falcon, USA) until 90% confluent. Tenfold dilutions of the virus suspension (10^{-1} – 10^{-8}) in DMEM maintenance medium were prepared. The growth medium was removed from the well plate. Then, 50 µl of virus suspension of each dilution per well (10^{-1} – 10^{-8}) was inoculated into sets of four wells of Vero cells, and incubated at 36ºC for 1 h. After this, 100µl of DMEM maintenance medium was added into each well, and the plate was incubated at 36ºC for 7 days. Four uninoculated wells of Vero cells served as negative controls. At 7 days post-infection (dpi), all the wells were examined for characteristic cytopathic effects, and the number of infected and uninfected cell cultures at each virus dilution recorded. The virus infectivity was determined using the Karber method.[15]

CV-A16 infection in mice

Three groups of mice (n=5 each) which were 1, 7 and 14-day old, were subcutaneously inoculated with 50µl and 100µl of CV-A16-N132 ($CCID_{50}$= 3.56 x 10^6/ml) respectively. Only half volume was given to the 1-day old group due to the size limitation of the animals and to minimize the risk of leakage. Two additional mice were mock-infected with phosphate-buffered saline (PBS) and served as controls for each group. After inoculation, the mice were frequently monitored several times daily for signs of infection up to 14 dpi. Mice were euthanized by isoflurane inhalation as soon as they developed severe signs of disease (e.g. paralysis). All animal experiments were approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, University of Malaya (Ethics number: 2013-12-03/PATHO/R/WKT).

Histopathology studies

The mouse tissues were fixed in 10% neutral buffered formalin for 3-5 days and trimmed into 5-7standard cross-sectional blocks as described previously[16], so that most tissues can be examined. 4µm thick tissues sections were cut from routinely processed, paraffin-embedded tissues and mounted on 3-aminopropyltrietoxysilane-coated slides, dried overnight, and stained with haematoxylin and eosin (H&E) for light microscopy. Immunohistochemistry (IHC) was performed using a previously described protocol with slight modifications.[16] Briefly, endogenous peroxidase in tissues was blocked, followed by antigen retrieval (30minutes, Tris-EDTA buffer, pH 9.0) and normal goat serum blocking. An Enterovirus Blend mouse monoclonal antibody (Catalogue number 3321, Light Diagnostics™, Merck Millipore, USA), diluted 1:100 or an anti-Enterovirus 71 rabbit polyclonal antibody[17], diluted 1:1000, was applied on tissues and incubated overnight at 4˚C. This was followed by horseradish peroxidase (HRP)-conjugated secondary antibody (ENVISION; Dako, Denmark) incubation at room temperature for 30 minutes, and 3,3'-diaminobenzidinetetrahydrochloride (DAB) chromogen, haematoxylin counterstaining and mounting in dibutyl phthalate and Xylene (DPX).

RESULTS

CV-A16 infection in mice

All the 1-day old and 7-day old ICR mice were susceptible to CV-A16 infection. From 3 to 4 dpi in the 1-day old group, and 5 to 6 dpi in the 7-day old group, signs of infection were observed, including reduced mobility, limb weakness or paralysis progressing to a moribund stage (Figure 1A). No paralysis or other signs of infection were observed in 14-day old mice even after 14 dpi. All the mock-infected mice remained healthy throughout the experiments.

Pathological findings

Many IHC-positive neurons were detected mainly in the brain stem (Figure 1B) and anterior horns of the spinal cord (Figure 1C) in infected animals. However, inflammatory changes were very mild. There were no obvious histopathological abnormalities in other brain areas such as cerebral cortex, thalamus, hypothalamus, hippocampus and cerebellum.

Skeletal muscles showed massive and widespread fibre necrosis (Figure 1D), involving muscles from head, facial, neck, shoulder, paraspinal and pelvic areas but inflammation was relatively mild. The distribution of viral antigens (Figure 1E) correlated well with muscle necrosis. In addition, many foci of antigen-positive adipocytes were observed in the brown fat (Figure 1F) around interscapular areas, mediastinum, kidney and spleen. No inflammation, necrosis or viral antigens were observed in skin, heart, lung, liver, kidney, pancreas and gastrointestinal tract.

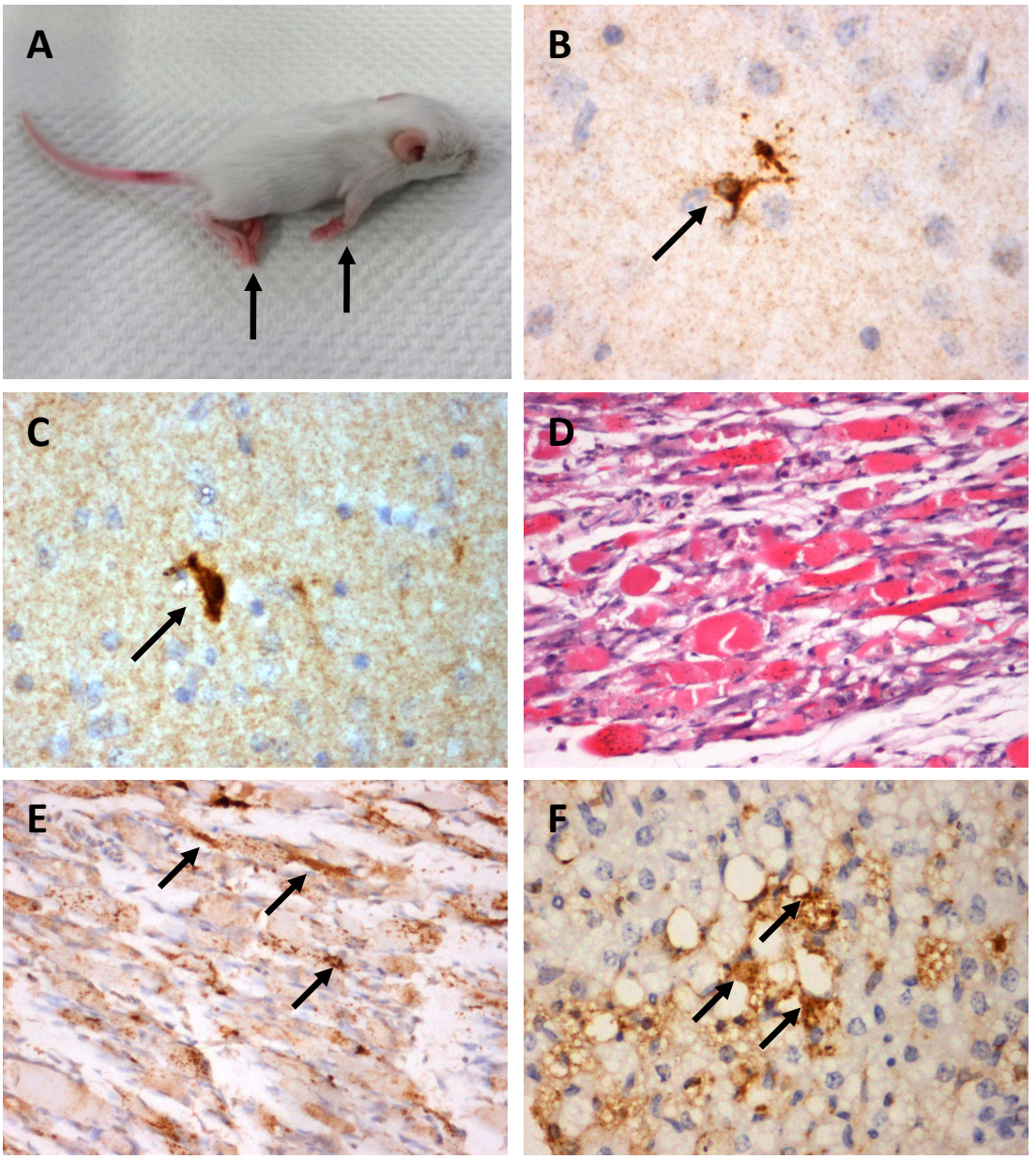

Figure 1. A 7-day old infected ICR mouse exhibited fore and hind limb paralysis (arrows) on 5 dpi (**A**). Histopathological findings in CV-A16-N132 infected mice: viral antigens were exhibited in neurons of the brain stem (**B**), spinal cord (**C**), skeletal muscles (**E**) and brown fat (**F**) in IHC stained with DAB. The limb skeletal muscles showed severe necrosis and mild myositis (**D**) with haematoxylin and eosin stain. Original magnification: 20x (D, E); 40x (C, F) and 60x (B).

None of the mice developed histological evidence of pulmonary oedema, myocarditis, skin or oral lesions. No viral antigens were detected in the mock-infected animals. The 14-day old mice tissues were not analyzed.

DISCUSSION

CV-A16 infection in our mouse model was age-dependent, with 100% of the 1-day and 7-day old mice showing signs of severe infection, while all 14-day old mice were not infected. Infected mice were observed to have reduced mobility, limb weakness and paralysis. Death/moribund stage occurred at 3-4 dpi in 1-day old mice and at 5-6 dpi in 7-day old mice. Age-dependant susceptibility to different CV-A16 strains has also

been observed in previous studies.[18,19] In one study, groups of neonatal mice (n=8~10) were inoculated at different ages via intracerebral inoculation with a CV-A16 strain (1.9 x 10^4 PFU/mouse), and their results showed that all the mice from1-day, 3-day and 5-day old groups developed signs of infection and death. Although all the 7-day old group mice were infected, 44.4% of the mice gradually recovered.[18] In another study, groups of 1-day, 2-day and 7-day old mice were also compared for susceptibility to another CV-A16 strain infection via intraperitoneal inoculation ($10^{6-7}CCID_{50}$), and their results showed that 95%, 100% and 8% of the mice, respectively, developed signs of infection.[13] To date, only one mouse-adapted CV-A16 strain, derived from a clinical strain, was able to cause nearly 80% of mortality in 14-day old mice.[19]All these findings supported the notion that age is a limiting factor for CV-A16 infection in mouse models. In the present study, our CV-A16-N132 strain, a non-mouse adapted virus was able to uniformly infect 7-day old mice but not 14-day old mice.

The observed signs of infection and pathological findings appear to be similar in 1-day and 7-day old mouse groups. Most skeletal muscles, showed widespread of CV-A16 infection causing necrotizing myositis, consistent with previous findings.[13,18] Neurons in the brain stem and spinal cord were infected confirming CV-A16 neurotropism. Thus paralysis in the model could possibly be caused by both myositis and neuronal infection. Why neuronal infection was not detected in other CNS areas has to be further investigated. Other studies have shown increase of viral titers in the CNS following infection but in our opinion, the pathological confirmation of CNS infection has not been convincingly documented previously.[12,13,20] Viral antigens were also observed in brown fat suggesting their involvement in viral replication, as has been reported in other enterovirus infections, including EV-A71 and coxsackievirus B.[16, 21, 22]

Although CV-A16 and EV-A71 are similar in many aspects, e.g., sharing the same Scavenger Receptor Class B, Member 2 (SCARB2) viral receptor, a close genetic relationship, and causing the clinically-indistinguishable HFMD[23,24], neurological complications are far less common and even less understood in CV-A16 infection. In the mouse model at least, a clinical isolated CV-A16 strain, from a patient with an uncomplicated HFMD was able to cause neuronal infection. Thus, our preliminary findings suggest that our mouse model could be a useful model for neuropathogenesis studies.

DISCLOSURE

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Conflict of interest: None.

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