The formation process of button ulcers in pigs experimentally infected with a subgenotype 2.1 isolate of classical swine fever virus

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ABSTRACT. We evaluated the role of classical swine fever virus (CSFV) in the formation of button ulcers in the mucosa of the gastrointestinal tract. Histopathological and immunohistochemical analyses of pigs experimentally infected with a subgenotype 2.1 isolate of CSFV, which was isolated in Japan in 2019, revealed follicular necrosis in the submucosal mucosa-associated lymphoid tissue and herniation of crypts as factors that contribute to the development of button ulcers during CSFV infection. These findings indicate that CSFV induces follicular necrosis and is one of the causative agents of button ulcers in pigs.

KEY WORDS: button ulcer, classical swine fever virus, herniation of crypt, histopathology

Classical swine fever (CSF) is a highly contagious viral disease caused by CSF virus (CSFV), which belongs to the genus *Pestivirus* in the family *Flaviviridae* [6]. CSFV isolates are categorized into three genotypes with three to four subgenotypes [2, 9]. CSF is characterized by an acute, subacute, chronic, or persistent course. Clinical signs are nonspecific and include fever, depression, anorexia, and constipation followed by diarrhea. Leukopenia is also reported to be one of the characteristic signs of CSF [1, 3]. As monocytes and macrophages are the main targets of CSFV, and altered monocytic cell function affects the vascular and immune systems, pathognomonic symptoms are dominated by the hemorrhagic syndrome and immunosuppression [7]. Gross pathological lesions are sometimes absent in acute cases, but marbled red lymph nodes, hemorrhages on the serosal and mucosal membranes of abdominal organs, such as renal petechiae and spleen infarction are typically found. In subacute and chronic cases, button ulcers may also be observed in the mucosa of the gastrointestinal tract, epiglottis, and larynx [1, 6].

In Japan, there have been no outbreaks of CSF since 1992, but it re-emerged in September 2018, and CSF cases have been continuously reported in wild boars and domestic pigs for more than two years. The CSFV isolated from these cases was classified into subgenotype 2.1, which was closely related to a CSFV isolate recently identified in China [8, 11]. This isolate seemed to have lower pathogenicity than that of isolates identified until 1992, which were virulent strains. Kameyama *et al.* [5] identified multifocal infarction of the margin of the spleen and button ulcers in the colon after inoculation with the JPN/1/2018 strain of CSFV; however, these lesions were not observed in all cases. In our experimental study that analyzed the pathogenicity of a subgenotype 2.1 isolate of CSFV designated Gifu-18-2019-NVAL01, which was isolated in Japan in 2019, we also found a button ulcer in an infected pig at necropsy.

Button ulcers are considered one of the pathognomonic changes in CSF [14]. However, the development of button ulcers in pigs infected with CSFV and the role of CSFV in their formation remain unclear. In this report, we aimed to demonstrate the development of button ulcers in experimentally CSFV-infected pigs and understand the process by which these ulcers are generated.

Three specific-pathogen-free pigs were purchased from the Central Research Institute for Feed & Livestock of National Federation of Agricultural Cooperative Associations (ZEN-NOH, Ibaraki, Japan). All animals were orally inoculated with 1.0 ml of culture

medium containing CSFV ($10^{7.0}$ TCID$_{50}$/ml of Gifu-18-2019-NVAL01). After viral inoculation, clinical signs, body temperature and the number of white blood cells were monitored for two weeks. All procedures were carried out in accordance with the Code of Practice for housing and care of animals used in the National Veterinary Assay Laboratory of Ministry of Agriculture, Forestry and Fisheries, which followed the Act on Welfare and Management of Animals, Standards relating to the Care and Keeping and Reducing Pain of Laboratory Animals, and other related regulations in Japan. On post-inoculated days (PID) 14 or 15, pigs were humanely euthanized, then subjected to postmortem examination. Collected tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 3 to 5 μm. For histopathological evaluation, these sections were stained with hematoxylin and eosin. To detect CSFV antigen, sections were incubated with an anti-CSF monoclonal antibody (WH303, APHA Scientific, Addlestone, UK) and stained using the Universal Immuno-

Fig. 1. A button ulcer found in the ileocecal area.

enzyme Polymer method with a Histofine simple stain Max PO (M) kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions.

Pigs developed pyrexia (>40°C) from PID 4 and leukopenia (<1.0 × 10^4 cells/mm^3) from PID 7. Although they showed visible clinical signs such as depression and anorexia, all three pigs survived throughout the experimental period. Necropsy observations showed that one pig had a 1–2 cm wide button ulcer in the ileocecal area (Fig. 1), whereas the others had no remarkable lesions in their gastrointestinal tracts. Histopathological observations showed that all three pigs had herniation of crypts to different extents, indicating that the mucosal epithelium fell into the submucosal mucosa-associated lymphoid tissue (MALT) as well as ulcers in the mucosa of the jejunum, ileum, and ileocecal area. Briefly, we observed that the follicular necrosis and moderate herniation of crypts occurred due to a prominent reduction of the cellular population following follicular necrosis in the submucosa of the ileum (Fig. 2A). At this lesion, there was no crypt epithelial cell necrosis. We also observed several lesions where the falloff became severe (Fig. 2B) and herniated crypts were dilated by hypersecretion in the mucosa of the jejunum, ileum, and ileocecal area (Fig. 2C). Additionally, exudative neutrophils gathered into the dilated intestinal crypt lined by flattened epithelial cells, leading to crypt abscess development by accumulation of exudative neutrophils, cell debris, and mucus in the mucosa of the jejunum and ileum area (Fig. 2D). Adjacent crypt abscesses fused together into massive necrotic lesions with the accumulation of mucus, cell debris, inflammatory exudate and bacterial colonies were also observed (Fig. 2D). At that lesion, almost all crypt epithelial cells were atrophic, necrotic, and/or absent in the lesion, and the accumulated mucus and inflammatory exudate erupted to the surface of the mucosa. Two or more such lesions were observed in each pig, whereas the only pig that developed the button ulcer showed severe ulcers with a pseudo-membrane mainly composed of exudative fibrin and mucus adjacent to the developing crypt lesions in the ileocecal area (Fig. 2E). The crypt lesion distribution was consistent with that of areas near the MALT, and more crypts were multifocally and severely affected in the lower regions of the small intestine. Immunohistochemical analyses revealed that all pigs had CSFV antigen in the follicular necrosis area in the submucosa adjacent to the region of herniated crypts and ulcers (Fig. 3). Conversely, CSFV antigen was not observed in the epithelial cells or areas with crypt abscesses and severe necrosis.

In this study, we identified differential progression of crypt herniation into the submucosal MALT, showing severe to moderate follicular necrosis and small to large ulcers on the lesions of crypt abscesses in the jejunum, ileum, and ileocecal mucosa in pigs infected with CSFV. Moreover, CSFV antigen was found only in the areas showing follicular necrosis. It has been reported that a button ulcer caused by CSFV is histologically characterized by mucosal ulceration and caseous necrotic centers that are composed of necrotic epithelia, bacteria, and detritus in lymphoglandular complexes [12, 15]. CSFV replicates in macrophages present in lymphoid tissues and indirectly causes apoptosis of lymphocytes through the expression of pro-apoptotic cytokines [13]. Therefore, follicular necrosis in CSF-infected pigs may result from these apoptotic lymphocytes. Additionally, chemical mediators released from activated macrophages can induce intestinal epithelial cell necrosis [12]. Hence, we hypothesized that the lesions observed in the present study showed different stages of formation of button ulcers from moderate lesions, which supported sequential pathogenesis wherein CSFV infection of macrophages induces follicular necrosis followed by herniation of crypts into the intestinal submucosa; crypt abscesses develop through accumulation of intestinal contents in the herniated crypts, and expanded abscesses cause necrosis in the surrounding mucosal epithelial tissues. Cytokines from activated macrophages might accelerate epithelial cell necrosis, and the characteristic button-like appearance of ulcers results from eruption of the crypt abscesses.

The results of this study suggested that follicular necrosis in the submucosal MALT and crypt herniation are triggers for the development of button ulcers during CSFV infection in pigs. CSFV induces follicular necrosis and hence, can be one of the causative agents of button ulcer formation in pigs. To our knowledge, this is the first report to demonstrate the progression of button ulcers following CSFV infection histologically and immunohistologically. Moreover, our findings demonstrate that a button ulcer in the mucosa of the gastrointestinal tract is one of the characteristic lesions following infection of the recently identified subgenotype 2.1 isolates of CSFV like traditional CSFV isolates. It is difficult to diagnose CSF only by antemortem inspection
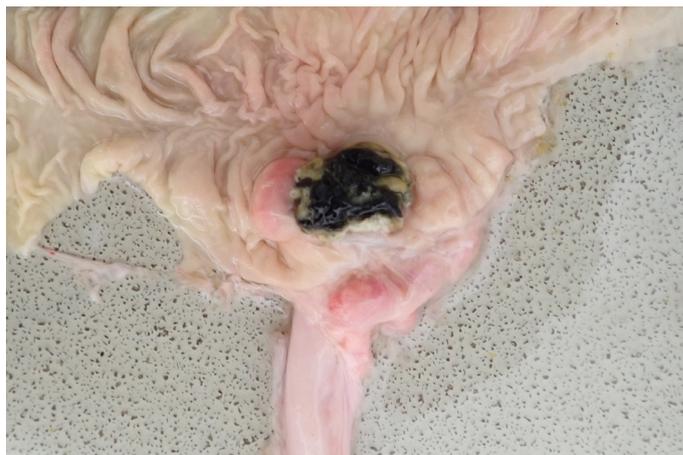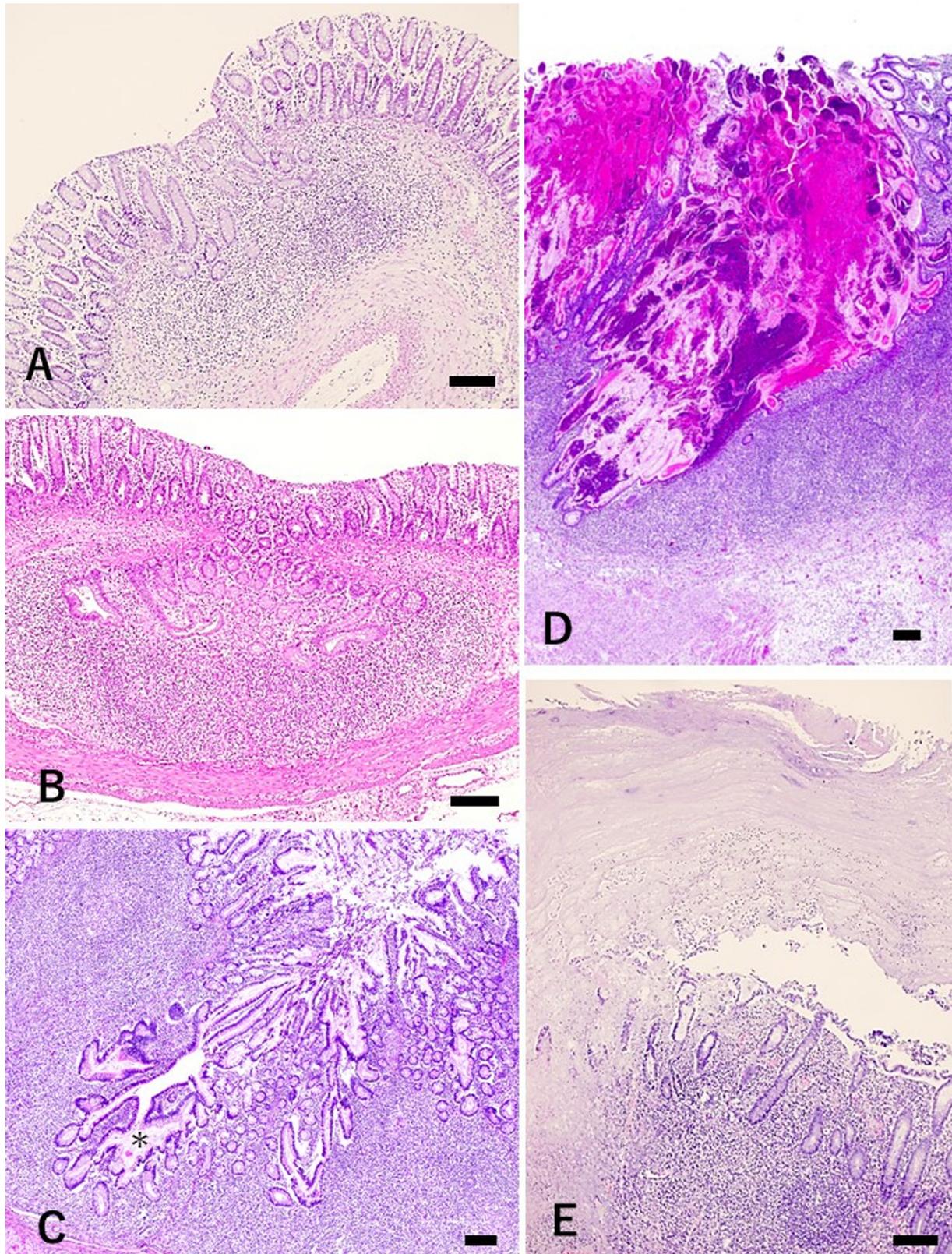
Fig. 2. Histopathology of the progression from herniation of crypts into the submucosal mucosa-associated lymphoid tissue to button ulcers. A
& B: Follicular necrosis and herniation of crypts (A: mild, B: progressed). C: Herniated crypts are dilated by hypersecretion. Secretions are
marked (*). D: Crypt abscesses fuse together and develop into massive necrotic lesions with accumulation of mucus, cell debris, inflammatory
exudate, and bacterial colonies. E: Severe ulcers with pseudo-membranes mainly composed of exudative fibrin and mucus form a "button ulcer".
A, B and, E were obtained from a pig that developed the button ulcer, and C and D were obtained from the other pigs as representative images.
Hematoxylin and eosin staining. Bar: 160 μm.
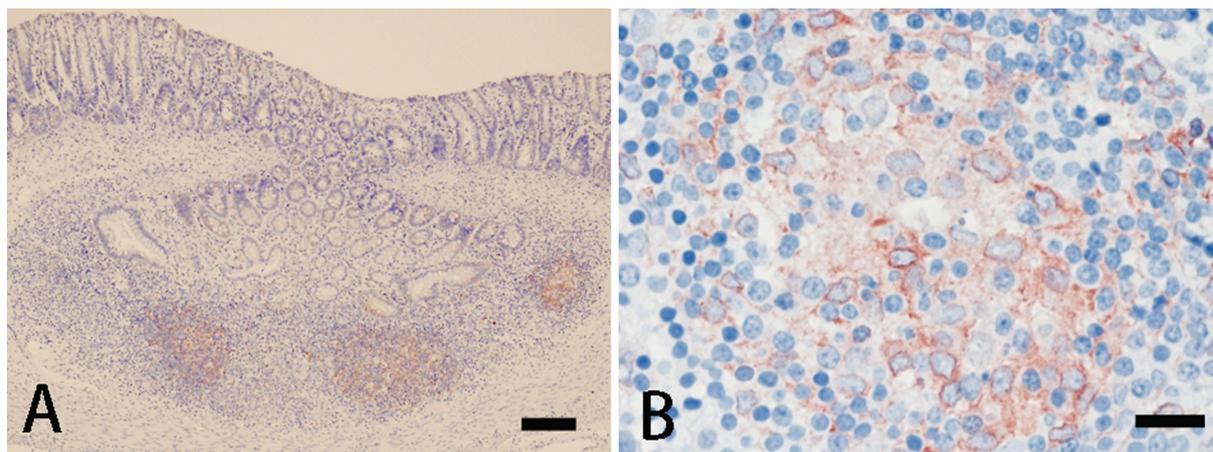
Fig. 3. Detection of classical swine fever virus (CSFV) antigen by immunohistochemistry. A: CSFV antigen is consistent with the follicle but is absent in the herniated crypt epithelium. B: High magnification of A. Immunohistochemistry for CSFV antigen. Bar, A: 180 µm, B: 20 µm.

because characteristic CSF lesions do not always appear in all CSF-infected pigs [10] and button ulcers in pigs are found not only in CSF but also in other diseases, including Salmonellosis [4]. Therefore, molecular and serological laboratory tests are required in addition to antemortem inspection to diagnose CSF. Even in this diagnostic strategy, this study supports that button ulcers are one of the pathognomonic changes during CSF, and laboratory tests should be performed when this change is detected at necropsy for rapid diagnosis of CSF.

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