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Virus–host interactions in persistently FMDV-infected cells derived from bovine pharynx

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ABSTRACT

Foot-and-mouth disease virus (FMDV) produces a disease in cattle characterized by vesicular lesions and a persistent infection with asymptomatic low-level production of virus in pharyngeal tissues. Here we describe the establishment of a persistently infected primary cell culture derived from bovine pharynx tissue (PBPT) infected with FMDV serotype O1 Manisa, where surviving cells were serially passed until a persistently infected culture was generated. Characterization of the persistent virus demonstrated changes in its plaque size, ability to grow in different cell lines, and change in the use of integrins as receptors, when compared with the parental virus. These results demonstrate the establishment of persistently infected PBPT cell cultures where co-adaptation has taken place between the virus and host cells. This *in vitro* model for FMDV persistence may help further understanding of the molecular mechanisms of the cattle carrier state.

Introduction

Foot-and-mouth disease virus (FMDV) is the type species of the Aphthovirus genus within the Picornaviridae family. The positive-stranded RNA virus has a genome of approximately 8400 nucleotides. FMDV causes a highly contagious and economically important disease in cloven-hoofed animals, including cattle, pigs, sheep and goats, characterized by vesicular lesions in the mouth and on the feet, teats and nares (Arzt et al., 2011; Grubman and Baxt, 2004). An important characteristic of the virus is its ability to establish a persistent infection (the so-called "carrier state") in up to 50% of both vaccinated and non-vaccinated cattle, with asymptomatic low-level excretion of virus from the pharyngeal region, which has also been described as the primary site of replication during the acute infection (Arzt et al., 2010; Burrows et al., 1971; Pacheco et al., 2010)

The virus may persist in the esophageal pharyngeal (OP) region of cattle and other ruminants (Salt, 2004; Sutmoller and Gaggero, 1965; Van Bekkum et al., 1959) for several years (Condy et al., 1985) without causing signs of disease. The importance of carrier animals in the epidemiology of FMDV has been a matter of debate for many years. Although the risks that FMDV carriers pose to susceptible animals are not well understood, control and eradication programs are complicated by the occurrence of the "carrier state" (Alexandersen et al., 2002; Knowles and Samuel, 2003; Ruiz-Saenz et al., 2009).

The mechanisms that mediate the establishment and maintenance of persistence *in vivo* are almost completely unknown. It has been previously suggested that the mechanisms for the establishment of viral persistence within the host may be mediated by changes in the virus, including the production of defective interfering particles, temperature-sensitive mutants, virus recombination and the infection of cells of the immune system with the concomitant alteration of their function (Salt, 1993). Alternatively, persistence may be the result of a dynamic equilibrium between the host immune response and the selection of viral antigenic variants (Gebauer et al., 1988).

A number of studies have demonstrated the importance of the tissues in the pharyngeal area during acute and persistent FMDV infection (Arzt et al., 2010; Burrows et al., 1971; Pacheco et al., 2010). In persistently FMDV-infected cattle, infectious virus and viral RNA have been isolated from oropharyngeal fluid samples (Burrows et al., 1971) and bovine pharyngeal epithelia (Prato Murphy et al., 1994), respectively. It also has been shown that FMD viral RNA was localized within epithelial cells of the soft palate and pharynx during persistent infection, suggesting that persistence *in vivo* results from the combination of a very specialized target epithelial cell population in the pharynx along with a

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specific cellular response, probably involving interferon and cytokine production (Zhang and Kitching, 2001). It has also been hypothesized that during persistence a cytokine response may be involved in delaying or preventing infection of susceptible cells, perhaps allowing clearance of the virus (Alexandersen et al., 2002). To date, no mechanism has been experimentally shown to be responsible for the establishment, maintenance or resolution of the carrier status for FMD.

Study of the mechanisms regulating activities between virus and host in persistently infected animals is complicated due to the innumerable virus and host factors influencing the interaction. Alternatively, the establishment of an *in vitro* model for FMDV persistence could elucidate molecular mechanisms that mediate evasion of host defenses and improve our understanding of the carrier state. FMDV-persistent cultures have been established using primary calf kidney cells by treating the cultures with anti-FMDV hyperimmune serum obtained from tissues of convalescent cattle, including bovine pharynx (Dinter et al., 1959; Mohanty and Cottral, 1971; Seibold et al., 1964). These studies demonstrated the persistence of FMDV in the cell cultures for approximately 25 weeks, although no cell passages were performed to establish a persistently infected cell line.

Other studies performed *in vitro* have focused on persistently infected cell lines derived from non-susceptible FMDV species (such as the baby hamster kidney cell line, BHK-21 or IBRS2) and using plaque-purified laboratory-adapted strains of FMDV (de la Torre et al., 1985). It was suggested that during serial passage of the culture, a co-adaptation of persistent virus and the host cells occurred, as reflected by an increased resistance of the cells to re-infection when challenged with the parental virus and an enhanced infectivity of the persistent virus for naive BHK-21 cells (de la Torre and Domingo, 1988; de la Torre et al., 1989).

In this study, we report the establishment of an *in vitro* persistently FMDV-infected primary cell culture derived from bovine pharynx tissue (PBPT), a relevant bovine tissue as a primary site of virus replication. The cells that survived the cytolytic infection were subcultivated until persistence was established. Characterization of the virus recovered from the persistent culture as well as characterization of the persistently infected cells suggests that genetic and phenotypic variations that develop during persistence may indicate a co-adaptation of virus and host cells. The establishment of this persistently FMDV-infected *in vitro* cell culture using a tissue critical during *in vivo* infection in natural hosts may provide a model that allows co-adaptation of both the virus and primary cells to study mechanisms of induction and reactivation of FMDV in carrier animals.

Results and discussion

Characterization of bovine pharynx cell cultures

Non-infected PBPT cell cultures were phenotypically characterized at different passage numbers using direct immunofluorescence and monoclonal antibodies (MAb) specific for critical cell markers. Basically, FMDV integrin receptors, ($\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_V\beta_6$), an epithelial cell marker (cytokeratin) and a non-epithelial cell marker (vimentin) were used for defining cell phenotypes present in these cell cultures. Low passage cell cultures were positive for all integrins, most cells were cytokeratin positive ($>80\%$), and less than 20% of the cells was positive for vimentin. In contrast, at passage number 15, cells remained positive for all integrins except $\alpha_V\beta_6$, a low percentage of cells was cytokeratin positive (5–10%), and the majority of the cells ($>80\%$) expressed vimentin (Fig. 1A). These results suggest that the number of non-epithelial type cells increases with increasing passage number while the epithelial-type cell population decreases.

Establishment of primary bovine pharynx cultures persistently infected with FMDV

To establish the *in vitro* persistent culture, monolayers of PBPT cultures were infected with the parental FMDV O1 Manisa (O1Manisa-par) at an MOI of 1 pfu/cell. At 24 h post-infection (hpi), approximately 80% of the cells exhibited CPE (Fig. 1B). The cells that remained attached were provided with fresh medium and regenerated a complete monolayer after 5–7 days of culture. Cultures were subsequently passed by detaching the cells using trypsin at intervals of 5–7 days. No evident CPE was observed from passage 2 up to passage 23 when persistently infected cultures were compared with the uninfected PBPT cultures (Fig. 1B).

The presence of virus during the serial passages of FMDV-infected bovine pharynx cells was analyzed by real-time reverse transcription PCR (RT-PCR) and IHC. The quantity of FMDV RNA detected by real-time RT-PCR varied with different cell culture passages. Ct values corroborate virus yields detected by plaque assay (Fig. 1C). Infectious virus was present with titers fluctuating between 10^3 and 10^5 pfu/mL in the supernatant of cell cultures from passages 2 to 23. FMDV antigens, analyzed by IHC (using a specific MAb against viral structural protein VP1), were constantly detected in cell cultures between passages 2 and 23. IHC of cell cultures at passage 15 demonstrated the presence of FMDV antigens in approximately 10% of the cells. Previous reports on persistently infected cultures of BHK-21(de la Torre et al., 1985; Donn et al., 1995; Martin-Acebes et al., 2010), IBRS-2 (de la Torre et al., 1985) or calf kidney cell (Dinter et al., 1959) also showed low percentages (0.05–10%) of infected cells without evidence of cytopathic effect. These results indicate that the bovine pharynx culture infected with FMDV O1Manisa-par became persistently infected and continues to harbor FMDV without showing evident cytopathic changes through 23 passages. Additional experiments used persistently FMDV-infected bovine pharynx cultures that had gone through at least 15 passages unless otherwise indicated.

Characterization of persistently FMDV-infected bovine pharynx cells

To determine the nature of the pharynx cells undergoing persistent infection with FMDV, a double-IFA staining and confocal microscopy were performed using MAb to the viral capsid protein (VP1) as well as MAb to different cellular markers (cytokeratin, integrin $\alpha_V\beta_6$ and vimentin). As stated earlier, after 15 passages only a low percentage (approximately 10%) of cells in the persistently infected PBPT cultures stained positive for viral antigen (Fig. 2A). VP1 was distributed as granules with perinuclear localization. Interestingly, none of the infected cells appeared positive for cytokeratin or integrin $\alpha_V\beta_6$ (data not shown) but were positive for vimentin (Fig. 2B).

To further characterize the localization of structural viral protein VP1 in persistently infected cultures, double-label IFA and confocal microscopy were utilized with monoclonal antibodies to identify different intracellular structures and pathways (Fig. 2B): actin and α-tubulin as markers for cytoskeleton; clathrin heavy chain and caveolin-1 as markers for clathrin and caveolae endocytosis pathways, respectively disulfide isomerase (PDI) as a marker for endoplasmic reticulum; Golgi zone area MAb to detect the Golgi apparatus; Rab11 as a marker to identify recycling endosomes; proteosomes as a marker to determine if virus is degraded in these structures. Results demonstrated that there is no colocalization between FMDV VP1 and most of the cell structures analyzed (Fig. 2B).

It is generally accepted that persistently FMDV-infected cattle harbors the virus in the pharynx and dorsal soft palate which are characterized by a highly specialized, non-cornified, and stratified squamous epithelia different from the surrounding epithelia