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Subcellular Immunolocalization of Porcine Circovirus Type 2 (PCV2) in Lymph Nodes from Pigs with Post-weaning Multisystemic Wasting Syndrome (PMWS)

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Summary

Post-weaning multisystemic wasting syndrome (PMWS) is one of the most significant porcine diseases worldwide. The causative agent is porcine circovirus type 2 (PCV2), the smallest virus known to infect animals. Data related to the structural and ultrastructural aspects of this infectious disease are sparse and there is little knowledge of the subcellular localization of PCV2 and its replication in the tissues of pigs naturally affected by PMWS. The present study describes the cellular localization of PCV2 in the lymph nodes of pigs affected by PMWS by application of immunolabelling techniques for light and transmission electron microscopy (TEM). PCV2 particles were exclusively detected in histiocytes. Ultrastructural alterations including marked dilatation of rough endoplasmic reticulum and swelling of mitochondria were associated with PCV2-labelled intracytoplasmic inclusions (ICIs) with recognizable virions. Within the ICIs icosahedral virus-like particles were specifically labelled with a PCV2 capsid antibody, whereas particles with a granular appearance were not labelled. Colocalization studies with confocal microscopy and double immunolabelling with TEM indicated a close relationship between virus and the mitochondria, suggesting that these organelles may play an important role in the replication of PCV2. The present findings further support the hypothesis that virus replicates within the histiocytes of lymph nodes.

Keywords: immunolabelling; PCV2; PMWS; ultrastructure

Introduction

Post-weaning multisystemic wasting syndrome (PMWS) is a multifactorial disease of pigs that is characterized by growth retardation, weight loss, laboured breathing, fever, diarrhoea and death in weaned piglets (Madec *et al*., 2008). During the last decade, PMWS and other porcine circovirus diseases (PCVDs) have been diagnosed in all swine-producing areas worldwide and they are now considered to be one of the most serious pig health concerns for producers (Segalés *et al*., 2005). The infectious agent of PMWS is porcine circovirus type 2 (PCV2), the smallest virus known to infect animals. Microscopically, diseased pigs show severe depletion of lymphocytes and histiocytic infiltration of lymphoid tissues, with high concentrations of PCV2 in lymphoid and non-lymphoid organs (Segalés and Domingo, 2002). Immunohistochemistry (IHC) and *in-situ* hybridization techniques for detection of PCV2 (McNeilly *et al*., 1999; Rosell *et al*., 1999) have been widely used to establish the laboratory diagnosis of PMWS (Segalés *et al*., 2005).

PCV2 is a non-enveloped, single stranded circular DNA viral pathogen of swine that measures 12–23 nm in diameter and is a member of the family Circoviridae, genus *Circovirus* (Allan *et al*., 1998; Onuki *et al*., 1999; Allan and Ellis, 2000; Mankertz *et al*., 2000; Mori *et al*., 2000; Rodríguez-Cariño and Segalés, 2009). The PCV2 virion has an icosahedral

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structure, described as $T = 1$, containing 60 capsid protein elements arranged in 12 pentamer-clustered units that protrude slightly and give an overall diameter of about 20.5 nm (Crowther *et al.*, 2003). Several transmission electron microscopy (TEM) studies have reported arrangements of paracrystalline arrays of small virus particles, approximately 17 nm in diameter, in epithelial and histiocytic (monocyte/macrophage) cells (Ellis *et al.*, 1998; Sato *et al.*, 2000; Imai *et al.*, 2006). However, most aspects of the subcellular distribution of virus in tissues from pigs naturally affected by PMWS are not yet known.

Several cell types may contain PCV2 nucleic acid and proteins, but histiocytic cells are the main site of virus localization (Ellis *et al.*, 1998). However, it is unclear whether PCV2 replicates in macrophages (Gilpin *et al.*, 2003), since the virus appears to require an actively dividing cell (Shibahara *et al.*, 2000) and macrophages are mostly non-dividing, terminally differentiated cells (Mohr *et al.*, 2009). In contrast, the internalization of PCV2 into monocyte/macrophage lineage cells is very effective and occurs by clathrin-mediated endocytosis (Misinzo *et al.*, 2005). It has recently been demonstrated that a low proportion of macrophages may support virus replication *in vivo* (Pérez-Martín *et al.*, 2007).

A previous TEM study revealed significant ultrastructural alterations in the lymph nodes of pigs naturally affected by PMWS. Such damage was mainly detected in histiocytes, which showed severe swelling and proliferation of mitochondria and contained intracytoplasmic inclusion (ICI) bodies with virus-like particles (VLPs) of 8–17 nm diameter arranged in paracrystalline arrays. ICIs were often found adjacent to markedly swollen mitochondria and icosahedral VLPs were observed inside these mitochondria. Viral factories (VFs) were found, suggesting that viral replication probably occurs within macrophages and that mitochondria might play a role in this process (Rodríguez-Cariño and Segalés, 2009). Taking into account the limited ultrastructural studies on PCV2 and PMWS-affected tissues, the objectives of the present work were to determine the localization of PCV2 particles at the subcellular level in the lymph nodes of pigs naturally affected by PMWS and to evaluate the relationship between potential PCV2 replication and cellular organelles using light and electron microscopy techniques.

Materials and Methods

Animals and Samples

Lymph nodes were obtained from six animals from a previous study (Rodríguez-Cariño and Segalés, 2009). Four pigs had been diagnosed as PMWS (wasting and respiratory distress, severe lymphocyte depletion and granulomatous inflammation in lymphoid tissues and a high concentration of PCV2 nucleic acid in those tissues) and two control animals had no PMWS-like lesions and no evidence of PCV2 infection.

Samples (cutted in block of 1 mm^3) of mediastinal and superficial inguinal lymph nodes were fixed in 4% paraformaldehyde (w/v) and 0.1% glutaraldehyde (v/v) (EM grade; Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (PB; Sigma, Steinheim, Germany) for 2 h. Subsequently, tissue blocks were washed with PB twice and stored in paraformaldehyde 2% (w/v) in PB at 4°C until further processing.

Sample Processing

Selected tissues were rinsed four times with PB and subsequently with glycine 20 mM (Riedel-dehaën, Seelze, Germany) and then cryoprotected by passage through a graded series of sucrose solutions of 0.7, 1.4 and 2.3 M in PB (Sigma). Samples were then frozen in liquid propane and stored in liquid nitrogen. Hydrated samples were immersed in methanol (Merck) containing 0.5 uranyl acetate for 72 h at −90°C, washed with methanol and embedded in Lowicryl HM20 resin (Polysciences Inc., Warrington, USA). Resin polymerization took place by ultraviolet exposure for 48 h at −45°C followed by an additional 48 h at 25°C. The dehydration and substitution processes were performed in a Leica EMAFS automatic freeze substitution system (Leica Microsystems GmbH, Wetzlar, Germany). Semithin (1.5 μm) and ultrathin (70 nm) sections were prepared with a Leica ultracut UCT microtome.

Immunogold Labelling for TEM

Ultrathin sections were placed on TEM gold-grids of 200 mesh. Immunolabelling of PCV2 was performed with a murine IgG2a monoclonal antibody specific for PCV2 capsid protein (Ingenasa, Madrid, Spain). Briefly, grids were blocked for 25 min in 1% bovine serum albumin (BSA; Sigma) in phosphate buffered saline (PBS) containing glycine 20 mM (Riedel-dehaën) and incubated with the monoclonal antibody at a dilution of 1 in 10 in 1% BSA/PBS for 2 h at room temperature. Grids were then washed four times in 1% BSA/PBS. Subsequently, sections were incubated with secondary goat anti-mouse antibody (at a dilution of 1 in 25) coupled with 10 nm gold particles (British BioCell International, Cardiff, UK) in 1% BSA/PBS for 40 min at room temperature. Grids were washed four times with PBS and then five times