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Review article

Ultrastructural pathogenesis of the PRRS virus

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Abstract – Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) was first isolated in swine alveolar macrophages (SAMs) and has subsequently been reported to replicate in other cell lines. Entry of the virus inside the cell takes place by receptor-mediated endocytosis. Following the entry of the virus into the cell, several not completely understood changes take place. PRRSV has been reported to be an apoptotic-inductor virus both in vivo and in vitro. Interestingly, it has been suggested that PRRSV-induced apoptosis occurs in cells other than those in which PRRSV replicates by a bystander mechanism. In this paper the ultrastructural pathogenesis of PRRSV will be reviewed.

PRRSV / apoptosis / cell pathogenesis

Résumé – **Pathogenèse liée à l'ultrastructure du virus du syndrome dysgénésique et respiratoire porcin.** Le virus du syndrome dysgénésique et respiratoire porcin (PRRSV) a été isolé pour la première fois dans des macrophages alvéolaires porcins (MAP), puis sa réplication a été mise en évidence dans d'autres lignées cellulaires. L'entrée du virus dans la cellule se produit par endocytose, par l'intermédiaire d'un récepteur. Des changements qui ne sont pas encore bien compris se produisent suite à cette pénétration. Le PRRSV a été considéré comme inducteur d'apoptose, à la fois in vivo et in vitro. Il a été suggéré que l'apoptose induite par le PRRSV se produit dans les cellules autres que celles dans lesquelles PRRSV se réplique, par un mécanisme d'action indirect et à distance. La pathogenèse ultrastructurale du PRRSV est le sujet de la présente synthèse.

syndrome dysgénésique et respiratoire porcin / virus / apoptose / pathogenèse cellulaire

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1. INTRODUCTION

The etiological agent of the Porcine Reproductive and Respiratory Syndrome, PRRSV, is related in its nucleotide sequence, genomic organisation and replication strategy to a group of small, enveloped, positive-stranded RNA viruses including the murine lactate dehydrogenase elevating virus (LDV), equine arteritis virus (EAV) and simian haemorrhagic fever virus, which are presently classified within the family *Arteriviridae*, order *Nidovirales* [8].

The PRRSV particle is 50 to 65 nm in diameter with a central nucleocapsid of approximately 30 to 35 nm in diameter [5]. The genome of PRRSV is a single-stranded 15 kb long polyadenilated RNA containing eight open reading frames (ORFs) [29]. Among the structural genes of PRRSV (ORFs 2 to 7), ORF7 is known to code for the nucleocapsid protein, and ORFs 2, 3, 4, 5 and 6 code for envelope proteins [30].

PRRSV isolates can be classified into two distinct serotypes, the American and the European serotype [3]. Distinction between both serotypes is substantiated by serological differences [3] and sequence data [17].

2. CELL SUSCEPTIBILITY

PRRSV was first isolated in swine alveolar macrophages (SAM), also being able to replicate in endogenous porcine cells in monocytes, glial cells and porcine pulmonary intravascular macrophages. In vitro, the virus has been able to be grown in two MA-104 subpopulations (monkey kidney cells) known as Cl-2621 and MARC-145 [4, 18, 51, 54]. PRRSV has a preference for replication in alveolar macrophages coming from pigs less than 6-weeks-old and within immature or recently activated macrophages [9, 27]. In fact, the number of alveolar macrophages susceptible to infection with PRRSV is apparently inversely proportional to the age of the pig from which the macrophages are collected [27].

Also, by immunocytochemistry or in situ hybridization, viral antigen or RNA has been found in macrophages of multiple tissues such as monocytes, endothelial cells, smooth muscle cells and fibroblasts [20]. The favorite cells for virus replication however continue being SAM of young animals [27].

3. HOW DOES THE VIRUS ENTER THE CELL?

During electron microscopy of virus and cell interaction carried out in 1996 [19], it was speculated that since the direct fusion of the PRRSV envelope with the cellular membrane was not observed at any time, PRRSV entry most probably occurs by receptor mediated endocytosis. In 1998, this hypothesis was confirmed [12] and a PRRSV receptor was identified on SAM by generation of SAM-specific monoclonal antibodies (MAbs). Two antibodies were generated that were able to block PRRSV infection on SAM by binding to molecules that are used for virus attachment on the surfaces of SAM [12].

A protein with a molecular mass of approximately 210 kDa was specifically precipitated by both MAbs. Since this 210 kDa protein is expressed only on the cell membranes of PRRSV-permissive SAM and not on those of non-permissive cells, it is suggested that this protein may determine the unique cell and tissue specificity of the virus [12].

4. MORPHOGENESIS OF PRRSV

Several morphogenesis studies of PRRSV have been done using both European and American strains of PRRSV [11, 38] in SAM and MARC-145 cells [19, 33]. Following binding to the cell receptor, virus particles are internalised by a microfilament-dependent process through

small clathrin-like coated vesicles. During this stage, an acidic pH is required to trigger a fusion event between the viral envelope and the endosomal membrane allowing the viral nucleocapsid to enter the cytoplasm for replication [19]. After penetration inside the SAM, viral antigen was detected in the cytoplasm at 6 hours post-infection (pi). At three hours pi, the first signs of degeneration took place, pinching off small vesicles with a double membrane from degenerated mitochondria. At 6 hrs pi, the nucleocapsid bud at the smooth endoplasmic reticulum and the lumen of the endoplasmic reticulum contained enveloped virus particles [38]. Virus containing vesicles were detected in the Golgi system and in the cytoplasmic region between the Golgi and the cell membrane. PRRSV may be released by exocytosis or by cell lysis.

Among PRRSV N, M and E proteins (encoded respectively by ORFs 7, 6 and 5), only the membrane associated E protein is glycosylated [30]. In order to study the synthesis and processing of the main proteins encoded by the structural genes of PRRSV in MARC-145 infected cells, Mardassi et al. synthesised monospecific antisera raised against *E. coli*-expressed ORF5, ORF6 and ORF7 products [25]. Shortly after synthesis, M-E heterodimer formation occurs suggesting that M protein accumulates in the ER and interacts herein with the E protein. In this sense, E molecules are slowly transported into the premedial Golgi compartment. After that, association of M and E proteins takes place forming disulfide linked heterodimers that are incorporated into virions preceding viral budding in pre-Golgi [25]. Virions seem to mature between the ER and the medial Golgi. Budding and accumulation of enveloped particles can only be observed between smooth-walled vesicles of the ER and Golgi apparatus [11]. Assembly of the virus is completed by interaction with the N protein that is accumulated in the cytosol. Vesicles containing enveloped nucleocapsids are derived from the ER and transported to the premedial Golgi where budding takes place, and the virus is released [25].

After virus release, dissemination in various organs might take place via infected monocytes and macrophages [54].

5. TOPOLOGY OF PRRSV PROTEINS INSIDE THE CELL

Replication of PRRSV has been described to be restricted to the cytoplasm. By the use of a specific monoclonal antibody against the nucleocapsid protein (N) of PRRSV in different virus susceptible cells, it was observed that the localization pattern of the protein is not limited to the cytoplasm of the infected cells but also to discrete regions of the nucleus. By the use of a specific anti-nucleoli polyclonal antibody, the subnuclear localization of the protein was demonstrated to be in the nucleoli. The gene product of open reading frame 7 of an American strain of PRRSV was expressed by coinfection of culture cells with a vaccinia virus expressing the T7 RNA polymerase of the T7 bacteriophage (vTF7-3) and a recombinant vaccinia virus encoding the open reading frame 7 gene under the T7 promoter. With this system, the protein is expressed as a fusion protein containing a histidine tag on its amino-terminal end. Again, when the monoclonal antibody specific to the PRRSV nucleocapsid protein was used in an inmunofluorescence procedure to locate the protein in double vaccinia infected mammalian cells, the N protein was found not only in the cytoplasm of coinfected cells but also in the nuclei and associated with the nucleoli [47]. This result indicates that the N protein of PRRSV does not require other PRRSV proteins, or viral replication for its subcellular localization.

There are previous reports on transport into nucleoli of proteins encoded by RNA viruses replicating exclusively in the cytoplasm, for example the M protein of the vesicular stomatitis virus [24]; the M protein of the New Castle disease virus [36], the

rev protein of the caprine encephalitis virus [42] or the core protein of the dengue virus type 4 [50]. The role of the nucleolar topology of the PRRSV N protein is unknown. It is possible that the N protein could be involved in inhibition of host protein synthesis as described in other viral proteins with nucleolar localisation [36]. Since the N protein can be concentrated in the nucleolus, it may affect ribosome assembly or function, leading to inhibition of protein synthesis. These regulatory functions may be exerted by newly synthesised proteins not incorporated into nucleocapsids that then migrate into the nucleus where they accumulate in the nucleoli as described for the Semliki Forest virus [31].

A perinuclear fluorescence characteristic of proteins localised in the ER appears by immunofluorescence with an anti-E and anti-M sera. N fluorescence is more cytoplasmic and diffuse, although more intense near the perinuclear region [25].

Following cell fractionation experiments, M and E proteins were shown to be microsomal membrane-associated whereas N was essentially found in the cytosolic fraction. From these results, it was found that E and M are true integral membrane proteins and are not loosely associated to membranes [25].

6. PRRSV IS AN APOPTOTIC INDUCTOR VIRUS

Many virus genomes encode gene products that are able to modulate apoptosis, a morphologically distinct and highly patterned type of cell death which plays an important role in processes such as homeostasis and the elimination of damaged cells. Some viral products prevent apoptosis whereas others induce apoptotic cell death. The meaning of such mechanisms are not completely known, although the former could contribute to the persistence or could prolong the time available for maximisation of virus progeny yield by lytic replication in the organism; the latter may contribute to the cytopathogenic effects of these viruses [43]. Apoptosis of the host cell before the completion of the viral replication cycle may limit the number of progeny and the spread of infection. Genes encoding products which trigger and prevent the development of apoptosis can be found in the same virus and both in RNA and DNA viruses [34, 39].

In the course of the characterisation of the viral product encoded in ORF5 of PRRSV, it was observed that its expression in mammalian cells as a single PRRS protein led to a rapid-acting and intense cytotoxicity. This cell toxicity was shown to occur by induction of apoptosis, as indicated by nucleosome ladder formation, chromatin condensation and rRNA degeneration [46].

Apoptosis induction was also observed after infection of culture cells with an adapter PRRSV strain and after infection of SAMs with a PRRSV field strain. The fact that the ORF5 protein induced apoptosis could not be prevented by bcl-2 expression, suggests that it might be acting downstream in the programmed cell death activation cascade [46]. Several mutants have been generated inside the ORF5 encoded protein to identify which region(s) is involved in the apoptosis induction process. Preliminary results indicate that the 118 amino-terminal end of the p25 protein is responsible for apoptosis induction [13].

These results of in vitro apoptosis induction of PRRSV have since been corroborated in vivo by different authors [45, 48, 49].

In a study in which the cellular tropism of PRRSV in gonadal tissues was investigated, abundant germ depletion was observed due to apoptosis [48] as determined by histology and in situ massive DNA fragmentation (TUNEL). In acutely PRRSV infected boars, the authors found the presence of cells with apoptotic features. These cells appeared to be derived mainly from spermatocytes, although occasional degenerative spermatogonia were observed.

Degenerative changes in these cells included nuclear rupture, pyknosis and vacuolar degeneration, cytoplasmic condensation and fragmentation of chromatin. These histopathological findings have also been confirmed by TUNEL reaction with an intense specific staining in nuclei and nuclear fragments (apoptotic bodies) in PRRSV infected and not in control animals. Apoptosis reached two peaks on days 7 and 25 pi, decreasing by 30 to 60 days pi. No correlation on a cell to cell basis between PRRSV in situ hybridization (ISH) signals and apoptotic activity were observed, suggesting the existence of a bystander mechanism or that apoptotic induction took place before the virus could be detected in infected cells [48]. This mechanism may help explain why in gnotobiotic piglets experimentally infected with the virus, the PRRSV infected cells frequently appear in clusters and are not always associated with microscopic lesions [21].

The bystander mechanism suggested in 1997 by Sur et al., was confirmed in 1998 by the same author and others [45, 49].

Apoptosis in vivo was confirmed in the lung and lymphoid tissues of experimentally intranasally infected PRRSV pigs. Apoptosis was studied by DNA ladder formation, TUNEL and Electronic Microscopy (Chromatin compactation against the nuclear envelope in SAM). The results showed that virus infection-induced apoptotic cells were more abundant than PRRSV infected cells detected by immunohistochemistry. Again, the majority of apoptotic cells did not colocalize with PRRSV-infected cells, suggesting the presence of an indirect mechanism of apoptotic induction.

Apoptosis was also detected by TUNEL staining in alveolar macrophages, porcine intravascular monocytes and lymphocytes inside the lungs and lymph nodes of infected pigs [45]. Again, although viral nucleic-acid positive cells both in the lungs and lymphonodes were multifocal in contrast to TUNEL-positive cells that were scattered throughout the sections, an apoptotic induction in uninfected bystander cells was suggested.

The mechanism of PRRSV-induced apoptosis in bystander cells is not known, but it has been reported previously in other viruses such as HIV, SIV and human herpesvirus [14,16]. In HIV-infected children and SIV-infected macaques, apoptosis occurs predominantly in bystander cells and not in the productively infected cells themselves [14].

Apoptosis may be triggered by different mechanisms:

- Activated macrophages and lymphocytes are able to express Fas and Fas ligand and become sensitive to Fas mediated apoptosis. In HIV, it has been demonstrated that HIV infection in macrophages not only increases the surface expression of Fas, but also results in the "de novo" expression of FasL and could participate in lymphocyte depletion in HIV infected individuals [2]. An indirect apoptosis in HIV uninfected T cells mediated by antigen presented cells has been reported [15].
- It has been shown that PRRSV infection of macrophages is able to significantly decrease the production of superoxide anion by those cells [51]. This PRRSV reducing oxidative metabolism could be related to apoptosis induction of the virus, as has been reported for the Sindbis virus (SV). SV is able to activate apoptosis by reducing the intracellular superoxide levels on AT-3 prostate carcinoma cells defining a novel redox pathway by which viruses can trigger cell death [23]. A decline in intracellular hydrogen peroxide production was observed to be associated with apoptosis in aging induced neutrophiles, meaning that apoptosis of neutrophiles is in part mediated by oxidative stress [32].
- Local release of cytokines in infected cells (TNF, IL-1β, …) is able to induce

apoptosis. In the course of PRRSV infection an increase in the production of some cytokines such as IL-1 β has been described. In human herpesvirus 6, the existence of a soluble protein produced by infected cells that could predispose uninfected bystander cells to apoptosis has been suggested [16].

- Since the p25 protein is an envelope protein that is included on the viral particle, the protein could produce this action as a constituent of the whole viral particle (not just by secretion).

Differences in the ability to induce apoptosis may explain the reported differences in virulence of PRRSV isolates [35]. Further studies are needed to determine the involvement of these or other mechanisms.

7. PERSISTENT INFECTION PRODUCED BY PRRSV

PRRSV possesses properties common to the genus *Arterivirus*: preferential replication in macrophages and the capacity to cause severe disease and persistence. LDV infected mice maintain virus persistence by the infection of new permissive macrophages combined with the ability of LDV to escape the host's defences [41]. In contrast to LDV, persistent PRRSV infection is not characterised by a persistent viraemia [37]. In fact, the persistence of PRRSV has been observed even though there appears to be a coincidence between the appearance of neutralising antibodies and the absence of viraemia. In most experiments, viraemia is detected from the second to third week post-infection, although viraemia of up to 6 to 7 weeks in duration has been reported [56]. There is no consensus on the exact duration and site of PRRSV replication during the persistent stage.

Although PRRSV was initially described as an acute infection causing respiratory distress in young animals and late-term abortions, the virus was recently demonstrated to establish persistent infections. Today, two stages can be considered: an *acute stage* including the first 2 weeks of infection during which maximal virus titers are recovered from all susceptible organs throughout the body, and a *persistent stage* characterized by lower levels of virus replication only in some organs [53].

Several studies confirmed that PRRSV is a multi-organ system infection; PRRSV infected cells by ISH were found in the nasal turbinate, stomach, small intestine, spiral colon, heart, aorta, brain, kidney, thymus, spleen, tonsils and lymph nodes. Persistence should be suggested when the virus is isolated from the tonsils, spleen and lymphoid tissues but not the lungs [40]. Also the virus seems to remain in the lungs [27], being able to be isolated from SAM 9 weeks post-exposure [28]. After intranasal infection of pigs with PRRSV, the virus was able to be recovered from broncho-alveolar fluid up to day 49, 28 days after the last virus isolation in the serum [44].

Evidence for persistence has come from several publications. Persistent infection with PRRSV in experimentally infected pigs was shown by isolation of the virus from oropharyngeal samples up to 157 days after challenge (134 days after the last isolation of virus from the serum of this pig) [55]. It has been reported that during persistent infections, PRRSV replication is restricted to lymphoid tissue or resides in immune-privileged sites such as the testes; but it is absent in the lung tissue and alveolar macrophages [7]. In fact, the same authors claim that 100% of the pigs born live to gilts infected with PRRSV at 90 days gestation and surviving at 21 days of age became persistently infected, and that boars are more susceptible to infection than other adult pigs.

The generation of PI (persistently infected) piglets from sows infected late in gestation has also been reported by other authors [1, 6, 26]. Congenitally infected pigs with PRRSV (piglets born alive to females infected in late gestation) tend to be viremic for extended periods of time, even 11 weeks

after birth [26]. Fifty percent of the piglets, born alive to serologically naive gilts/sows having received intranasal inoculations at 85 to 90 days of gestation, were unthrifty, 88% of these piglets were viremic. Pigs alive for 5 to 14 days of age became thumpers and all liveborn pigs developed secondary *S. suis* infections between 6 and 14 days of age. Viraemia persisted until 7 weeks of age. In this study, the pigs that were euthanised between 60 and 130 days after birth, presented virus in the tonsils (by virus isolation) and selected lymph nodes (in situ hybridization/PCR). PRRSV does persist in pigs infected in utero and virus replication appears to exist in subpopulations of macrophages and other cells in the lymph nodes and tonsils. Viral RNA could also be detected in persistently infected pigs 210 days after birth. One animal was PCR serum negative for several weeks, then became positive 210 days after birth [6].

It was established that subpopulations of PI pigs exist in most swine herds with chronic PRRSV problems. These pigs serve as a source of virus, infecting naive littermates and being an important source of virus when commingled with serologically negative pigs in nurseries [6]. Re-excretion of PRRSV in pigs by exogenous corticosteroid treatment of movement stress has also been demonstrated [1].

PRRSV is also able to persist in the boar. After experimental infection, the presence of viral RNA in semen was detected by PCR on day 92 pi and viral isolation took place from the bulbourethral gland of one boar on day 101 pi [10].

This persistence capacity of PRRSV seems to be in disagreement with the apoptosis described ability of the virus. In this sense, differences in pathogenicity among different virus strains have been demonstrated under experimental conditions [35] and the existence of viral mutants affected in their apoptosis induction capability is not known. Other possible explanations may be that the expression of some cellular proteins could alter the interaction of the virus with its host cell, inducing a switch from lytic growth to persistence, as was reported in the Sindbis virus [22]. The presence of viral genes responsible for suppression of apoptosis in PRRSV cannot also be discarded as was described in other RNA viruses [52].

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