PORCINE ARTERIVIRUS ENTRY IN MACROPHAGES

Heparan sulfate–mediated attachment, sialoadhesin-mediated internalization, and a cell-specific factor mediating virus disassembly and genome release

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1. INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) was first described as a new disease in pig herds in North America late in the 1980s and beginning of the 1990s in Europe and is characterized by respiratory disease in young piglets and late-term reproductive failure.[1,2] The causative agent of the disease was identified in Europe in 1991 and in the US in 1992 as a virus, PRRS virus (PRRSV).[2-5]

PRRSV is classified with equine arteritis virus (EAV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) in the family *Arteriviridae*, which is grouped with the *Coronaviridae* and the *Roniviridae* in the order *Nidovirales*.[6-8] The virus is an enveloped particle with a diameter of 50 to 65 nm and contains a polyadenylated, positive-strand RNA genome.[9] The genome is about 15 kDa and encodes 9 open reading frames: ORF1a and 1b code for nonstructural proteins, ORF2a, 2b, 3, 4, 5 and 6 code for structural membrane proteins, and ORF7 codes for the nucleocapsid protein (N).[10-13]

Currently, PRRSV is present in most, if not all swine-producing areas of the world, including North and South America, Western and Eastern Europe, and Asia. As the virus is now enzootic in most countries, the number of acute disease outbreaks has diminished and infections are in general mild and subclinical.[14] From an economical point of view, however, the virus still causes major losses and is considered as the most important pig disease worldwide.

Characteristic for all members of the *Arteriviridae* is not only that they have a very narrow host tropism, but also that they share a marked *in vivo* tropism for cells of the monocyte/macrophage lineage.[14,15] Even in cell culture, arteriviruses have, with the exception of EAV, a very narrow cell specificity, only allowing replication in primary

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macrophages from their respective hosts and in a limited number of cell lines upon adaptation of the virus.[16]

For PRRSV, it has been shown that *in vivo*, the virus infects a subpopulation of resident macrophages present throughout the body, and that alveolar macrophages are primary target cells for the virus.[17, 18] *In vitro*, porcine alveolar macrophages (PAM) have been shown to efficiently sustain virus replication.[5, 19, 20] Peripheral blood monocytes can also be infected *in vitro* at very low levels, but only when they have been cultivated for 24 h.[19, 21] Interestingly, peritoneal macrophages were shown to be refractory for PRRSV infection.[19] Besides primary macrophages of porcine origin, only the African green monkey kidney cells MA-104, and cells derived thereof, such as Marc-145 cells, can sustain *in vitro* virus replication.[22] Although PRRSV has thus both *in vivo* and *in vitro* a very restricted cell tropism, it can replicate in several cell lines upon transfection of the genomic RNA, indicating that the restricted cell tropism is very likely the result of the presence or absence of specific receptors on the membrane of macrophages and of other macrophage-specific factors.[23, 24]

In our laboratory, we have been studying for several years PRRSV entry in macrophages, and two receptors on macrophages have been identified: (1) heparan sulfate and (2) sialoadhesin. The glycosaminoglycan heparan sulfate was identified as a receptor because both heparan sulfate and heparin, an analogue of heparan sulfate, strongly reduce infection of macrophages with both European and American PRRSV strains when present during virus inoculation.[25, 26] Other glycosaminoglycans, such as chondroitin sulfate A and dermatan sulfates had no effect on PRRSV infection, indicating that the observed effect was specific. Also, treatment of macrophages with heparinase I, an enzyme which destroys heparan sulfate, reduced infection of macrophages. Using flow cytometry and labeled PRRSV, it was observed that heparin strongly reduced virus attachment to macrophages.[26] Heparan sulfate has also been proposed as receptor on Marc-145 cells, because heparin reduces PRRSV infection when present during infection, or when these cells are pretreated with heparinase I.[27]

Sialoadhesin, a macrophage-specific protein belonging to the sialic acid-binding immunoglobulin-like lectin (Siglec) family, was identified as a PRRSV receptor on macrophages because (a) a sialoadhesin-specific monoclonal antibody (mAb 41D3) is able to completely block infection of macrophages, (b) mAb 41D3 reduces PRRSV attachment to macrophages, (c) PRRSV co-localizes with sialoadhesin on the surface of macrophages, (d) both the *in vivo* and *in vitro* PRRSV susceptible porcine cells express sialoadhesin, and (e) nonsusceptible cells internalize PRRSV upon expression of a recombinant sialoadhesin.[28-30]

In this overview, our findings about the different steps of PRRSV entry in macrophages (attachment, internalization and genome release into the cytoplasm) will be discussed and these will be related to the findings of others on PRRSV entry in both macrophages and Marc-145 cells.

2. PRRSV ATTACHMENT: INITIATED BY AN INTERACTION WITH HEPARAN SULFATE AND FOLLOWED BY ATTACHMENT TO SIALOADHESIN

The role of heparan sulfate and sialoadhesin in PRRSV attachment to macrophages was first evaluated using flow cytometric attachment studies in the presence or absence

of heparin and/or sialoadhesin-specific mAb 41D3, this to block respectively virus attachment to heparan sulfate, sialoadhesin, or both.[31] Both heparin and mab 41D3 reduced attachment up to a maximum reduction of respectively 83% and 50%. A combination of both heparin and mAb 41D3 could however completely block virus attachment. Clearly, from these results it can not only be concluded that both heparan sulfate and sialoadhesin mediate PRRSV attachment to the macrophage, but also that no other receptors are involved in this process. Furthermore, analysis of the attachment kinetics to macrophages showed that PRRSV first binds to heparan sulfate, followed by an interaction with sialoadhesin.[31]

3. PRRSV INTERNALIZATION IN MACROPHAGES IS A CLATHRIN-DEPENDENT PROCESS MEDIATED BY THE MACROPHAGE-SPECIFIC RECEPTOR SIALOADHESIN

Previously, it was shown that PRRSV internalization in macrophages is a clathrin-dependent process.[32] Recently, we identified the macrophage-specific receptor sialoadhesin as a receptor involved in PRRSV internalization, because expression of a recombinant sialoadhesin on the surface of cells which are nonpermissive for PRRSV infection, such as PK-15 or CHO cells, makes these cells capable of internalizing the virus.[28, 31] Furthermore, it was shown that the heparan sulfate receptor, which mediates virus attachment, is not necessary for internalization, as recombinant sialoadhesin internalizes PRRSV both in CHO cells expressing heparan sulfate and in CHO cells lacking heparan sulfate.[31] Because heparan sulfate is thus not essential for PRRSV attachment to sialoadhesin and subsequent sialoadhesin-mediated internalization, it is thought that the interaction with heparan sulfate serves to concentrate virus particles on the cell surface, which enhances virus binding to sialoadhesin, resulting in enhanced internalization and infection. Mouse and human sialoadhesins were previously described as a sialic acid binding lectin[33-35] and recently, we found that porcine sialoadhesin is also a sialic acid binding lectin.[36] We also observed that enzymatic removal of sialic acid from the PRRS virion surface almost completely blocked infection of macrophages, indicating that virus attachment to sialoadhesin is mediated by sialic acids present on one or more structural viral glycoproteins.[36]

PRRSV internalization in macrophages via sialoadhesin was shown to be a clathrin-mediated process[28,32] and although PRRSV internalization in Marc-145 cells is also clathrin-dependent,[37] our data indicate that sialoadhesin is not involved in PRRSV infection of Marc-145 cells and that another receptor is responsible for virus internalization in these cells. This conclusion is based on the fact that (a) sialoadhesin could not be detected on Marc-145 cells, neither using sialoadhesin-specific mAb 41D3, nor using a sialoadhesin specific polyclonal serum generated by DNA immunization in mice, (b) removal of sialic acid from the surface of PRRSV, which results in an almost complete block of PRRSV infection of macrophages, has no effect on infection of Marc-145 cells. Although an internalization receptor has not been identified on Marc-145 cells, it was suggested by preliminary studies that this receptor is a protein. Two monoclonal antibodies have been described which block PRRSV infection of Marc-145 cells and which recognize a yet unidentified 60–66 kDa Marc-145 cell surface protein.[38, 39]

4. VIRUS DISASSEMBLY AND GENOME RELEASE REQUIRES ENDOSOME ACIDIFICATION AND A CELL-SPECIFIC FACTOR

Previously, it was shown both for macrophages and Marc-145 cells that PRRSV is internalized via a clathrin-dependent process and that endosome acidification is essential for infectivity.[32,37] When endosome acidification was inhibited using lysosomotropic bases, no infected cells were detected using either confocal or electron microscopy up to several hours after inoculation. This was in contrast to a normal infection in which virus was disassembled after internalization.[32,37] From these results, it was concluded that an acidic pH is needed to trigger fusion between viral and endosomal membrane, which is a key element in virus disassembly and release of the viral genome in the cytoplasm.

Although we observed that PRRSV can be internalized in several cell types refractory for PRRSV infection upon expression of a recombinant sialoadhesin, a productive infection was not observed in these cells.[28,31] A comparison of the viral entry process in macrophages, Marc-145 cells and cells expressing recombinant sialoadhesin revealed that virus was disassembled upon internalization in macrophages and Marc-145 cells, while it remained present in endocytic vesicles in recombinant sialoadhesin expressing cells.[28] Apparently, productive infection not only requires a receptor such as sialoadhesin, which mediates virus internalization, but also a cell-specific factor mediating virus disassembly and genome release. Since it was previously shown that PRRSV also remains in endosomes upon internalization in macrophages and Marc-145 cells when the pH drop is blocked by drugs, we investigated if acidification of the extracellular medium could overcome this blockade in cells expressing recombinant sialoadhesin. Although this method was shown to be successful in the case of Marc-145 cells to counteract the effects of ammonium chloride,[37] it did not result in virus disassembly and productive infection in recombinant sialoadhesin expressing cells. So, while clearly endosome acidification is essential for infection, other cell-specific factors, such as fusion receptors or proteases, are also involved in PRRSV infection. Currently, experiments are being conducted to identify these co-factors involved in the post-internalization steps of PRRSV infection of macrophages.

Interestingly, two sets of monoclonal antibodies that reduce or block PRRSV infection of macrophages have been described.[40] One set recognizing a 220-kDa protein completely blocked infection. The nature of this protein has not been identified, but as suggested by Wissink et al. (2003)[40] and based on our recent results, this 220-kDa protein is likely to be the macrophage-specific protein sialoadhesin. Another set of monoclonal antibodies that recognizes a 150-kDa protein doublet was shown to strongly reduce, but not block PRRSV infection of macrophages. This protein is not identified, but could represent a receptor that is potentially involved in fusion between viral and endosomal membrane and subsequent release of the viral genome in the cytoplasm. Future studies using these monoclonal antibodies could clarify these issues.

5. ACKNOWLEDGMENTS

The authors would like to thank all the past and present members of our laboratory who have contributed to our understanding of the complexity of the entry process of this intriguing virus: Xiaobo Duan, Nathalie Vanderheijden, Gerald Misinzo, and Sarah Costers.

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