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Jonna Nykky

Virus-Cell Interactions as a Pathological Mechanism of Parvovirus Infection

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UNIVERSITY OF JYVÄSKYLÄ

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ABSTRACT

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Pathogenesis is the process by which an infection leads to disease. Virus-cell interactions determine the pathological consequences of viral infection on a cellular level. In this thesis the interactions of canine parvovirus (CPV), a small non-enveloped DNA virus, with its host cell were deciphered. These interactions account for the cytopathic effect and the pathology seen in infected hosts. Research for this thesis demonstrated that after viral entry depolarization of mitochondrial membrane potential and production of reactive oxygen species were observed, indicating mitochondrial stress. However, these indicators of mitochondrial stress normalized at 6 h post infection, due to the activation of cellular survival signalling involving activation of ERK1/2 signalling cascade. Normalization of mitochondrial homeostasis prevented death of host cells early in the infection and enabled viral replication. Later in the infection, after beginning of viral replication as detected with expression of NS1 protein, CPV was found to arrest the progression of cell cycle at S phase supporting viral replication. Furthermore, activation of caspases 8, 9 and 3/7 was detected during infection. Damage to the mitochondrial membrane, plasma membrane and DNA indicated involvement of the intrinsic apoptotic cell death pathway. In addition, CPV was shown to interact with mitochondrial outer membrane throughout the infection. However, apoptotic changes appeared only after the beginning of viral replication pointing to the involvement of other factors than direct virus-mitochondria-interactions in inducing apoptotic changes. Parvoviral infection was additionally observed to affect the structure of host cells. Long tubulin containing protrusions were observed in CPV infected cells. These protrusions may have a role in egress of viruses providing a means for virus to spread from cell to cell. Results obtained in this thesis add knowledge to the multiple virus-cell interactions that could occur and to the mechanisms of how a parvovirus infection causes disease.

Keywords: Apoptosis; canine parvovirus; cell cycle arrest; cell signalling; mitochondria; pathology.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-III.

I Nykky J., Tuusa J. E., Kirjavainen S., Vuento M., Gilbert, L. 2010. Mechanisms of cell death in canine parvovirus-infected cells provide intuitive insights to developing nanotools for medicine. *International Journal of Nanomedicine* 5: 417–428.

II Nykky J., Vuento M, Gilbert L. 2014. Role of mitochondria in parvovirus pathology. *PlosOne* 9: e86124.

III Pakkanen K., Nykky J., Vuento M. 2008. Late steps of parvoviral infection induce changes in cell morphology. *Virus Research* 137: 271–274.

RESPONSIBILITIES OF JONNA NYKKY IN THE ARTICLES OF THIS THESIS

Article I: I designed the experiments with Leona Gilbert and Matti Vuento. I was responsible for the majority of the experiments. Jenni Tuusa performed the studies with human cells. I and Sanna Kirjavainen conducted the experiments with electron microscopy. I wrote the article together with Leona Gilbert and Matti Vuento. I processed all the figures.

Article II: I was responsible for planning the article together with Leona Gilbert and Matti Vuento. I performed the experiments. I wrote the article together with Leona Gilbert. I processed all the figures.

Article III: I participated in the planning of the article with Kirsi Pakkanen and Matti Vuento. I was responsible for the studies with A72 cells. Kirsi Pakkanen conducted experiments with NLFK cells and wrote the article.

Study I was performed under the supervision of Docent Leona Gilbert and Professor Matti Vuento. Study II was performed under the supervision of Docent Leona Gilbert. Study III was conducted under the supervision of Professor Matti Vuento.

ABBREVIATIONS

ADV	Aleutian mink disease virus
Apaf-2	apoptotic protease activating factor 2
ATM	ataxia telangiectasia-mutated kinase
ATR	ATM- and Rad3-related kinase
Bcl	B cell lymphoma
BPV	bovine parvovirus
MVC	canine minute virus
CPV	canine parvovirus
CD	cluster of differentiation
COX IV	cytochrome c oxidase IV
CDK	cyclin-dependent kinase
DIC	differential interference contrast microscopy
DDR	DNA damage response
DNA-PK	DNA-dependent protein kinase
DPV	duck parvovirus
EBV	Epstain-Barr virus
ER	endoplasmic reticulum
ERK1/2	extracellular regulated kinase 1/2
FPV	feline panleukopenia virus
G	gap
GRIM-19	genes associated with retinoid/interferon–induced mortality 19
GPV	goose parvovirus
H-1PV	H-1 parvovirus
HCV	hepatitis C virus
HSV-1	herpes simplex virus 1
HBoV	human bocavirus
HCMV	human cytomegalovirus
HIV-1	human immunodeficiency virus 1
HPV	human papillomavirus
B19V	human parvovirus B19
HIF-1	hypoxia-inducible factor 1
JNK	c-Jun N-terminal kinase
LAMP	lysosomal membrane protein
MVM	minute virus of mice
MAM	mitochondria-associated ER membranes
MAPK	mitogen-activated protein kinase
M	mitosis
MTR	MitoTracker Red
NLFK	Norden laboratory feline kidney cell
NLS	nuclear localization signal
NP	nuclear phosphoprotein
NS	non-structural protein
ORF	open reading frame

PLA_2	phospholipase A_2
PI3K	phosphatidylinositol 3-kinase
pERK1/2	phosphorylated extracellular regulated kinase 1/2
PPV	porcine parvovirus
p.i.	post infection
PI	propidium iodide
ROS	reactive oxygen species
SV40	Simian virus 40
S	synthesis
STS	staurosporine
TNF	tumor necrosis factor
VP	viral protein
Vpr	viral protein R

1 INTRODUCTION

Viruses are small agents referred to as intracellular parasites since they are unable to proliferate outside a host cell. The simplest viruses consist of a protein shell that envelops the nucleic acid. They are reliant on their cellular host for providing the building blocks and the energy needed for their replication. Consequently, viruses are a burden to the host cell by exhaustion of its metabolism.

Viruses can infect all forms of life from bacteria to mammals, but they are highly specific in their targets. To be able to infect an organism the virus has to first gain entry to the organism and thereafter to the precise type of host cell. Pathogenesis is the process by which an infection leads to disease. Pathogenic mechanisms of viral disease include entry to the host organism, initial replication, spread to the main target organs where disease is induced and spreading of the virus into the environment. Infection may induce direct cell damage and cell death by interfering with cellular functions, or indirect cellular damage that results from integration of viral genome, induction of mutations, inflammation or via the action of host immune response e.g. by destruction of cells presenting viral antigens and by formation of immune complexes. Viral infections are a common cause of human diseases and new viral diseases are emerging. Viral infections are a less controlled threat to human health than bacterial infections that can in most cases by treated with drugs. Knowledge of viral pathogenic mechanisms is essential for correct diagnosis and treatment of diseases, for prevention of viral spread in the environment and for development of anti-viral drugs.

Aim of this thesis was to shed light to virus-cell interactions using canine parvovirus as a model virus. Parvoviruses cause diseases in animals and humans and are additionally being developed as a tools in medicine. The pathogenic mechanism of parvoviruses has to be described in detail in order to use them to treat diseases and also to have better treatment strategies of the viral infection itself. Results obtained in this thesis add knowledge to how parvoviruses interfere with cellular actions that may account to the diseases induced by infections.

2 REVIEW OF THE LITERATURE

2.1 Parvoviruses

Parvoviruses are small, nonenveloped viruses with single stranded DNA genome (Berns and Parrish 2007). Parvoviruses infect several species ranging from rodents to humans. According to report of the International Committee on Taxonomy of Viruses 2012 the *Parvoviridae* family is divided to two subfamilies, the Parvovirinae and the Densovirinae, and infect vertebrates and insects, respectively (Anon. 2013). The subfamily Parvovirinae is divided to five genera: *Amdovirus, Bocavirus, Dependovirus, Erythrovirus* and *Parvovirus* (Table 1). The subfamily Densovirinae is divided to 4 genera: *Brevidensovirus, Densovirus, Iteravirus* and *Pefudensovirus*. A new proposal to update the *Parvoviridae* taxonomy is under review (Cotmore *et al.* 2013) that suggests three new genera for the subfamily Parvovirinae and two new genera for subfamily Densovirinae. These are *Aveparvovirus*, *Copiparvovirus* and *Tetraparvovirus* in Parvovirinae, and *Hepadensovirus* and *Penstyldensovirus* in Densovirinae. Additionally, genus and species names are being updated in the proposal. Genus names are modified to contain parvo/denso in the name, and *Densovirus* is changed to *Ambidensovirus*. Species names are systematically changed and those will be different from the virus names; e.g. canine parvovirus will be included in the new proposed taxonomy to the genus *Protoparvovirus*, species *Carnivore protoparvovirus I* and the virus name will be canine parvovirus. This thesis concerns the subfamily Parvovirinae.

TABLE 1 The representative members of the subfamily Parvovirinae and their abbreviations (according to the report of International Committee on Taxonomy of Viruses 2012).

Genus	Species	Abbreviation
Amdovirus	Aleutian mink disease virus	ADV
Bocavirus	Bovine parvovirus	BPV
	Canine minute virus	MVC
	Human bocavirus	HBoV
Dependovirus	Adeno-associated virus 1–5	AAV 1-5
	Duck parvovirus	DPV
	Goose parvovirus	GPV
Erythrovirus	Human parvovirus B19	B19V
	Simian parvovirus	SPV
Parvovirus	Canine parvovirus	CPV
	Feline panleukopenia virus	FPV
	H-1 parvovirus	H-1PV
	Minute virus of mice	MVM
	Porcine parvovirus	PPV
	Rat parvovirus	RTPV

2.1.1 Structure of parvoviruses

The parvoviral capsid is 18–26 nm in diameter and their single stranded genome is 4 to 6 kb long. The genome has two open reading frames (ORF), one coding for structural viral proteins (VP) and other coding for non-structural proteins (NS) (Cotmore and Tattersall 1987, Reed *et al.* 1988). Separate from other parvoviruses, recently discovered bocaviruses have three ORFs. The middle ORF codes for nuclear phosphoprotein (NP1) unique for bocaviruses (Qiu *et al.* 2007). Organization of CPV, BPV and B19V ORFs are displayed in Figure 1. Parvovirus capsids contain structural proteins VP1, VP2 and VP3. However, B19V, ADV and bocavirus capsids are composed only of VP1 and VP2. For all parvoviruses VP1 and VP2 are alternatively spliced products of the same mRNA. VP1 contains the whole sequence of VP2 and an additional unique N-terminus that harbours a nuclear localization signal (NLS) and phospholipase A_2 (PLA_2) activity (Cotmore and Tattersall 1987, Reed *et al.* 1988, Vihinen-Ranta *et al.* 1997, Zadori *et al.* 2001). VP3 is generated by N-terminal cleavage from VP2 and is present only in DNA containing capsids (Paradiso *et al.* 1982, Tullis *et al.* 1992). Similarly to VP proteins, NS proteins are produced by alternative splicing from the same mRNA. They share the amino terminal sequence, but the sequence of the carboxy terminus is unique in both proteins (Cotmore and Tattersall 1986).

60 copies of VP proteins assemble to form an icosahedron with T = 1 symmetry (Fig. 1) (Cotmore and Tattersall 1987, Berns and Parrish 2007). The three dimensional structure of CPV was the first parvovirus capsid structure to be determined (Tsao *et al.* 1991). Thereafter, capsid structures of FPV, MVM,

PPV, B19V and HBoV have now been determined and shown to share similar features (Agbandje *et al.* 1993, Llamas-Saiz *et al.* 1997, Simpson *et al.* 2002, Kaufmann *et al.* 2004, Gurda *et al.* 2010). Protein subunits are typical antiparallel 8-stranded β barrels. Large loops connect the strands of barrels and form two thirds of the structure constructing most of the surface of a capsid. On the fivefold axis there is a cylindrical pore that is used for packing of DNA. Capsids have a depression at the twofold axis and a canyon around the fivefold axis (Tsao *et al.* 1991, Wu and Rossmann 1993, Xie and Chapman 1996). In addition, parvovirus capsid has a spike at the threefold axis (Tsao *et al.* 1991). The structure of B19V differs from other parvoviruses as B19V capsid does not have a spike at the threefold axis and the channel at fivefold axis is closed (Kaufmann *et al.* 2004). However, most variability in the parvovirus structure is seen in loops connecting the strands of β barrels. Loops form most of the surface of capsids and they determine host specificity and receptor binding (Parrish 1991, Parker and Parrish 1997, Simpson *et al.* 2002, Kaufmann *et al.* 2004, Gurda *et al.* 2010).

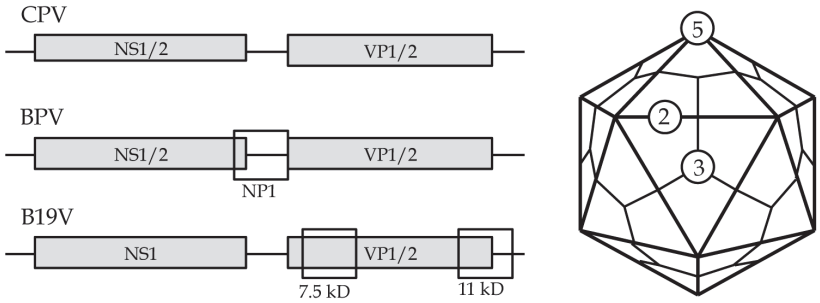

FIGURE 1 Structure of parvovirus genome and capsid. Schematic transcription maps of *Parvovirus* canine parvovirus, *Bocavirus* bovine parvovirus and *Erythrovirus* human parvovirus B19 are demonstrated. The left open reading frame (ORF) of CPV and BPV codes for non-structural proteins NS1 and NS2 and the right ORF codes for structural proteins VP1 and VP2. The nuclear phoshoprotein (NP1) of BPV is translated from the middle ORF. For B19V the left ORF codes only for NS1 while right ORF codes for VP1 and VP2. Two additional small NS proteins 7.5 kDa and 11 kDa are translated form small ORFs overlapping with ORF coding for VP1/2. Icosahedral parvovirus capsid consists of 60 copies of VP proteins. Numbers (2, 3 and 5) indicate axes of symmetry. Modified from Reed *et al.* 1988, Qiu *et al.* 2007, Heegaard and Brown 2002 and Tsao *et al.* 1991.

In addition to VP proteins, most parvoviruses have two non-structural proteins, NS1 and NS2. As an exception, bocaviruses have also a nuclear phosphoprotein NP1 (Qiu *et al.* 2007) while B19V has only NS1 and two small proteins named 7.5- and 11-kDa proteins (Heegaard and Brown 2002). NS1 is a multifunctional protein required for productive infection. It can bind to specific DNA motifs, acts as a transcriptional activator and it has ATPase, helicase and nickase activities (Gavin and Ward 1990, Christensen *et al.* 1995, Nuesch *et al.* 1995, Christensen and Tattersall 2002). NS1 is involved in initiation of viral DNA replication by binding to DNA and forming a single-strand nick after which it is covalently attached to the DNA. As NS1 is attached to DNA it presumably has a role in

encapsidation of DNA (Cotmore and Tattersall 1995). These multiple activities of NS1 are controlled by phosphorylation of the protein (Nuesch *et al.* 1998, Corbau *et al.* 1999). Because of its functions NS1 is highly cytotoxic (Moffatt *et al.* 1998, Corbau *et al.* 2000, Nuesch and Rommelaere 2006, Poole *et al.* 2006, Nuesch and Rommelaere 2007, Kivovich *et al.* 2010, Poole *et al.* 2011, Kivovich *et al.* 2012).

NS2 is involved in replication, capsid assembly and release of progeny from the nuclei (Naeger *et al.* 1993, Cotmore *et al.* 1997, Eichwald *et al.* 2002, Miller and Pintel 2002). It has been reported to be required for productive infection of MVM and H-1 viruses (Naeger *et al.* 1990, Li and Rhode 1991). However, CPV NS2 is not necessary for productive infection (Wang *et al.* 1998). Bocavirus NP1 protein is involved in viral replication and it interferes with signalling pathways to suppress production of interferon β to modulate innate immunity (Sun *et al.* 2009). Additionally, expression of NP1 induces cell cycle arrest and apoptosis (Sun *et al.* 2013). The small 11 kDa protein of B19V is essential for B19V infectious as it is needed for production of VP2 and for nuclear export of capsid proteins (Zhi *et al.* 2006). Additionally, 11kDa protein has been reported to induce apoptosis of B19V infected cells (Chen *et al.* 2010). The role of 7.5 kDa protein in B19V infection is unknown. Through their cytotoxic activities, parvoviral NS proteins account strongly for the pathology of parvoviral infections that are discussed later.

2.1.2 Parvovirus life cycle

Parvoviruses enter cell through receptor-mediated endocytosis (Fig. 2) (Harbison *et al.* 2008). Some receptors utilized for parvoviral entry are transferrin receptor (CPV and FPV), sialic acid (MVM, PPV) and globoside (B19V) (Cotmore and Tattersall 1987, Brown *et al.* 1993, Parker *et al.* 2001, Boisvert *et al.* 2010). Parvoviruses bind to their receptor on the cell surface and are taken in by clathrin-mediated endocytosis (Parker and Parrish 2000, Dudleenamjil *et al.* 2010, Boisvert *et al.* 2010). Cell entry through alternative pathways has been proposed (Harbison *et al.* 2008) and indeed, entry through macropinocytosis has been suggested for PPV alongside clathrin-mediated pathway (Boisvert *et al.* 2010). Inhibition of viral replication with drugs that prevent acidification of endosomes have indicated that acidification is required for viral escape into cytosol (Ros *et al.* 2002, Suikkanen *et al.* 2002, Suikkanen *et al.* 2003a, Dudleenamjil *et al.* 2010, Quattrocchi *et al.* 2012). Parvoviruses travel in endocytic vesicles to reach vesicles resembling late endosomes or lysosomes where low pH is suggested to induce conformational changes to the capsid and N-terminus of VP1 is externalized. This region of VP1 protein includes a phospholipase A2 activity that is believed to be important for escape from endocytic vesicles and it also contains the NLS (Vihinen-Ranta *et al.* 1997, Zadori *et al.* 2001, Vihinen-Ranta *et al.* 2002, Suikkanen *et al.* 2003a).

Experiments with MVM and PPV revealed that inhibition of the activity of proteasomes blocked the infection. These results suggest that MVM and PPV, after release to the cytoplasm, require activity of proteasome for successful replication (Ros *et al.* 2002, Boisvert *et al.* 2010). In the cytoplasm virus particles

are transported along microtubules towards the nucleus and they enter the nucleus apparently as intact capsids (Vihinen-Ranta *et al.* 2000, Suikkanen *et al.* 2003b, Harbison *et al.* 2008, Dudleenamjil *et al.* 2010). However, it has been reported for B19V that only viral DNA enters the nucleus while capsids remain in cytoplasm (Quattrocchi *et al.* 2012). It has been proposed that parvoviral entry into the nucleus may not be through the nuclear pore in spite of the reported NLS in parvovirus capsid proteins as MVM disrupted the nuclear membrane in infected mouse fibroblasts as well as when MVM was microinjected to *Xenopus* oocytes (Cohen and Pante 2005, Cohen *et al.* 2006). The mechanism involved in disruption of the nuclear envelope includes virus-induced relocalization of caspase 3 to the nucleus and cleavage of lamin B1 (Cohen *et al.* 2011). If nuclear entry of parvoviruses occurs through disruptions of the nuclear envelope, the NLS found in parvoviral proteins could have a role in the cytoplasmic targeting of synthetized proteins towards the nucleus (Boisvert *et al.* 2010, Cohen *et al.* 2011). Schematic representation of parvoviral entry is illustrated in Figure 2 showing endocytic transport from clathrin-coated pits to lysosomal membrane protein 2 (LAMP-2) positive vesicles resembling lysosomes and further transportation along microtubules to nucleus. Another entry pathway is suggested to be macropinocytosis. Some parvoviruses require activity of proteasomes before nuclear entry.

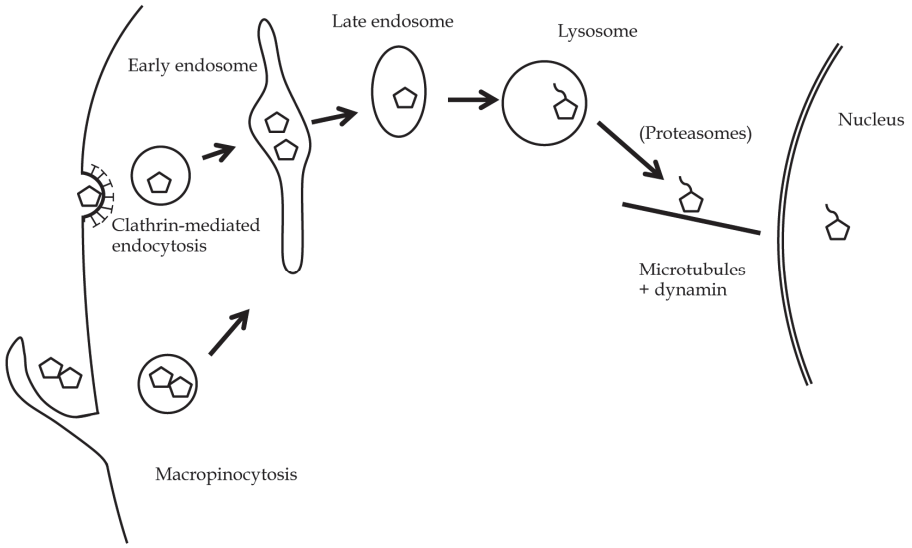

FIGURE 2 Entry of parvoviruses. Viruses bind to their receptor on the cell surface and are taken in by endocytosis. An alternative entry occurs through macropinocytosis. In endocytic vesicles viruses are transported to vesicles resembling lysosomes where acidic pH induces structural alteration to the capsid. From these vesicles viruses are released to the cytoplasm where capsids travel along microtubules to the nucleus. Proteasome may be included in the entry pathway of some parvoviruses. Modified from Harbison *et al.* 2008.

Replication of parvoviral DNA occurs in the nucleus. DNA of parvoviruses is linear, single stranded and it has terminal palindromic hairpins at both ends (Reed *et al.* 1988, Berns and Parrish 2007). Cellular proteins are utilized in parvovirus replication and the host cell has to go through S phase for successful replication (Berns and Parrish 2007). Parvoviruses replicate their DNA through rolling hairpin mechanisms where the hairpin formed by the 3′ end serves as a primer (Tattersall and Ward 1976, Cotmore and Tattersall 1995). The NS1 protein is covalently attached to the 5′ end of parvoviral genome during DNA replication and the nicking and helicase activities of NS1 are involved in the replication. NS1 is an early gene product and accumulation of NS1 terminates host cell DNA replication (Cotmore and Tattersall 1987).

Viral capsids assemble in the nucleus where translated VP proteins are transported from the cytoplasm (Yuan and Parrish 2001, Lombardo *et al.* 2002). DNA is incorporated into preassembled capsids with the help of NS proteins (Cotmore and Tattersall 1995). NS2 interacts with nuclear export factor chromosome region maintenance 1 (Crm1) to promote nuclear egress of assembled capsids and release of progeny has been thought to occur after cell death and lysis (Richards *et al.* 1977, Cotmore and Tattersall 1987, Eichwald *et al.* 2002, Miller and Pintel 2002, Nuesch and Rommelaere 2006). However, more recent reports propose parvoviral egress to be a regulated process. MVM NS1 protein interacts with casein kinase IIα that modifies gelsolin activity, resulting in modifications of actin cytoskeleton. Actin remodeling is involved in export of MVM containing vesicles (Bär *et al.* 2008). Assembled viral particles are engulfed into coat protein complex II vesicles at the perinuclear area and they travel through endoplasmic reticulum (ER) and Golgi. During egress viral capsids are modified by phosphorylations that increase viral infectivity. In addition, the transport of MVM through ER and Golgi somehow controls the cytolysis to provoke a more efficient release of progeny (Bär *et al.* 2013). From Golgi MVM is transported in LAMP-2 positive vesicles resembling late endosomes/lysosomes to the plasma membrane (Bär *et al.* 2008, Bär *et al.* 2013). As acidic environment in these vesicles has a role in the modification of capsids during entry (Suikkanen *et al.* 2003a) acidification may have a role also in maturation of viruses (Nuesch and Rommelaere 2006, Bär *et al.* 2008, Bär *et al.* 2013). How the egress of viruses through ER and Golgi controls the lysis of the cell and how capsids are modified in acidic vesicles remains to be studied.

2.2 Pathology of parvovirus infections

Although parvoviruses infect a plethora of species, most of them do not cause a disease in infected animals (Berns and Parrish 2007). Major parvoviruses causing disease in vertebrates include B19V, CPV, FPV, PPV, ADV, MMV, H-1PV, GPV and DPV. Infections vary from mild infections to fatal ones. The pathogenesis is commonly determined by the need for actively replicating cells and the target tissue. Due to the need of mitotic activity, young animals, or even foetuses, are

often infected. PPV and HBoV cause more severe infections when co-infection of some other virus occurs (Parrish 2008). Diseases caused by parvovirus infection include leukopenia, enteritis, myocarditis (CPV), panleukopenia (FPV), fetal infection and death (PPV), hepatitis (GPV), a chronic immune complex-mediated disease with respiratory symptoms (ADV), respiratory diseases (HBoV) and erythrocyte aplasia, erythema, polyarthtitis, arthralgia, transient aplastic crisis and fetal hydrops (B19V) (Csiza *et al.* 1971, Pollock and Carmichael 1990, Alexandersen *et al.* 1994, Zeeuw *et al.* 2007, Berns and Parrish 2007, Jartti *et al.* 2012). Pathogenesis of CPV and B19V has been examined more in detail and these are discussed below.

2.2.1 Pathogenesis of CPV infection

CPV infection occurs in dogs by oronasal route (Macartney *et al.* 1984). First clinical signs are seen about 4 days after exposure and these include fatigue, depression, vomiting and diarrhea (Pollock and Carmichael 1990). The initial replication site of CPV is the thymus from where it spreads to other lymphoid tissues. From lymphoid tissue the infection extends through blood stream to intestine where virus antigens can be detected from day 4 after oral inoculation. At 4 days after inoculation the virus can be isolated from the feces of infected animals (Macartney *et al.* 1984, Meunier *et al.* 1985). Antibody production can be detected 7 days after infection and the virus is rapidly eliminated from the serum and feces (Macartney *et al.* 1984). CPV replicates intensively in mitotically active cells of the intestine causing extensive damage to intestinal epithelia and symptoms of enteritis results. Viral antigens have additionally been detected in the spleen, liver and bone marrow. However, the main replication site of CPV is the lymphoid tissue and intestinal epithelium (Macartney *et al.* 1984). Myocarditis is also observed in CPV infected puppies. This type of infection may proceed without clinical signs until lethal heart failure. CPV infection induces myocarditis if puppies are infected near the time of birth. These infections are prevented by maternal antibodies and are rare nowadays (Pollock and Carmichael 1990). Extensive necrosis can be seen in histological samples from CPV infected dogs intestine and lymphoid tissue. Cytopathy of infection leads to enteritis, leukopenia and diminished lymphoid tissue (Macartney *et al.* 1984, Meunier *et al.* 1985) but the mechanism of cytopathy in CPV infected cells has not been studied thus far.

2.2.2 Pathogenesis of B19V infection

B19V is a widespread virus and infection occurs commonly during childhood. Up to 60 % of adults are positive for B19V IgG. The virus is transmitted through the respiratory route or via blood from mother to fetus (Heegaard and Brown 2002). B19V proliferates first in the throat and then spreads to bone marrow where erythroblasts are infected. 6 days after inoculation viremia can be detected with mild illness of influenza like symptoms and clinically non-significant haematological abnormalities. Viremia persists for about five days and it is

connected to substantial decrease in reticulocytes and milder decrease in neutrophils and lymphocytes. Three weeks after infection IgG levels rise leading to formation of immunocomplexes and a rash or arthralgia appear (Anderson *et al.* 1985, Kerr 2000). Furthermore asymptomatic infection is common or symptoms are connected to common flu (Heegaard and Brown 2002).

Erythema infectiosum (or fifth disease) is the most common symptom of B19V infection in children whereas arthralgia and arthritis are the most common symptoms seen in adults. However, arthritis is also frequently noticed in infected children (Heegaard and Brown 2002). If the mother is infected during pregnancy viruses can infect the fetus leading to hydrops fetalis and fetal death (Kerr 2000, Heegaard and Brown 2002). Infection in erythroblasts leads to apoptosis and reduction in haemoglobin. However, this is usually subclinical because red cells are not affected by B19V and antibody response rapidly neutralizes the virus. In immuno-compromised patients or patients with condition involving shortened red cell survival the outcome of B19V infection can be more severe (Kerr 2000). In addition to erythema infectiosum and arthritis B19V infection is connected to other symptoms and diseases. These include hematological manifestations, hepatitis, neurological syndromes and aplastic crisis in patients with hemolytic anemia (Kerr 2000, Heegaard and Brown 2002). Autoimmune diseases triggered by B19V infection include systemic lupus erythematosus and rheumatoid arthritis (Takahashi *et al.* 1998, Hemauer *et al.* 1999). B19V infection induces apoptosis in infected cells (Moffatt *et al.* 1998, Poole *et al.* 2004, Chen *et al.* 2010) that accounts for part of the pathological outcomes, but the mechanisms leading to different autoimmune diseases has not been resolved yet. The viral actions leading to pathological outcomes are discussed in the next section.

2.3 Mechanisms of viral pathology

The aim of viral pathology is to find out the processes how viral infections cause diseases in the host. Many factors have a role in determining the pathological outcome of the infection. These include how viruses enter the host and spread in the host, what is the tissue and cell specificity of the virus, what kind of damage the virus causes to the host cell and also how host's immune system reacts towards the infection. Viruses interact with cellular proteins and manipulate cellular pathways for their own benefit to be able to replicate and produce new viruses. Certain mechanisms of viral pathology are discussed below given emphasis on mechanisms studied in this thesis.

2.3.1 Metabolic changes observed during viral infection

The virus has to get the energy and metabolites required for a productive infection from the host cell. Viruses have means to alter the metabolic status of the host cell and create an environment favourable for the viral replication. Activation of glycolysis to increase ATP production has been reported during

human cytomegalovirus (HCMV), hepatitis C virus (HCV) and Sindbis virus infections (Munger *et al.* 2006, Ripoli *et al.* 2009, Silva da Costa *et al.* 2012). Metabolic shift to glycolysis induced by HCMV infection is achieved by activation of adenosine monophosphate activated protein kinase to reprogram the host cell metabolism (McArdle *et al.* 2012). In addition to activation of glycolysis, the tricarboxylic acid cycle and pyrimidine biosynthesis noticeably increased during the HCMV infection. On the other hand, the amount of metabolites related to the lipid metabolism and nucleosides decreased as they were used in the macromolecular synthesis of the HCMV (Munger *et al.* 2006).

The increased synthesis of viral macromolecules sets requirement for increase in energy production. Indeed, increased respiration capacity has been observed e.g. in HCMV, rubella, dengue and Sindbis virus infections (El-Bacha *et al.* 2007, Kaarbo *et al.* 2011, Silva da Costa *et al.* 2012, Claus *et al.* 2013). During HCMV infection a higher energy production is achieved through increased mitochondrial biogenesis by viral protein UL37 exon 1 and inhibition of apoptosis by viral RNA (Reeves *et al.* 2007, Kaarbo *et al.* 2011). This virally encoded RNA molecule of HCMV interacts with the complex I of electron transport chain to protect the mitochondrial membrane potential and production of ATP. Effect is mediated by interaction of viral RNA with Genes associated with retinoid/interferon–induced mortality 19 (GRIM-19) protein. The interaction prevents the relocalization of GRIM-19 observed after apoptotic stimuli (Reeves *et al.* 2007). Metabolic changes during viral infection enable viral replication and the pathological outcome of the infection. Knowledge of how viruses induce metabolic changes has increased and these actions of viral infection have been considered as a way to treat viral diseases with drugs (El-Bacha *et al.* 2007, Silva da Costa *et al.* 2012, Melnick *et al.* 2012, Claus *et al.* 2013).

2.3.2 Interference of viruses with cell signalling

To create the optimal environment for production of progeny, viruses are able to adjust the equilibrium of signalling pathways promoting cell survival or death. Pathological consequences of viral interference with cellular signalling pathways depend on the activated/inhibited signalling pathway. Virus induced signalling may lead to interference of cell cycle (uncontrolled proliferation or cell cycle block), cell death or improper immune response (Galluzzi *et al.* 2008, Chaurushiya and Weitzman 2009, Boyapalle *et al.* 2012, Li *et al.* 2013). Some of these signalling pathways and signalling molecules are discussed below.

The phosphatidylinositol 3-kinase (PI3K) is an enzyme controlling multiple downstream signalling pathways including the serine–threonine kinase Akt signalling pathway. PI3K/Akt pathway is involved in regulating cell survival, proliferation and inhibition of apoptosis, all important factors for viral survival (Cooray 2004, Piguet and Dufour 2011). In addition, Akt is involved in the regulation of glucose metabolism. Activation of PI3K/Akt pathway increases glucose uptake and production of ATP through glycolysis (Piguet and Dufour 2011). Epstein-Barr virus (EBV) is an oncogenic virus capable of transforming resting B cells to permanently growing cells. One factor contributing to this

transformation is viral latent membrane protein 1 that activates PI3K/Akt signalling inducing cell proliferation and enabling a latent infection (Dawson *et al.* 2003). Additionally, PI3K/Akt signalling is involved in acute viral infections. Rotavirus NS1 protein activates PI3K/Akt signalling in the beginning of the infection to suppress proapoptotic action of viral NS4 protein to gain time for viral replication (Bhowmick *et al.* 2012). Similar activation of PI3K/Akt signalling is reported during influenza A virus infection (Ehrhardt *et al.* 2007).

Mitogen-activated protein kinase (MAPK) signalling cascades, including extracellular signal regulated kinase (ERK1/2), p38 and c-Jun N-terminal kinase (JNK), mediate cellular responses to extracellular signals like growth factors, stress and inflammatory cytokines. Activation of MAPK cascades leads to proliferation, differentiation, immune response or cell death depending on the activated cascade (Fig. 3) (Piccoli *et al.* 2006, Roskoski 2012). ERK1/2 signal cascade promotes cell survival, proliferation and differentiation (Roskoski 2012). ERK1/2 cascade is involved in life cycle of many viruses, including human immunodeficiency virus 1 (HIV-1), influenza virus, HCMV and vaccinia virus (Yang and Gabuzda 1999, Johnson *et al.* 2001, Pleschka *et al.* 2001, Schweneker *et al.* 2012). Purpose of ERK1/2 signalling during viral infection is commonly to prevent premature apoptosis and ensure viral replication. Both increase and decrease of ERK1/2 activation has been reported in response to the B19V VP1 protein or a B19V infection, respectively (Tzang *et al.* 2009, Chen *et al.* 2011), but involvement of these signalling cascades in parvovirus infection has not been studied further. JNK and p38 cascades are involved in signalling for cell death and cytokine production (Pearson *et al.* 2001). Like ERK1/2 activation, activation of JNK or p38 pathways has been reported during many viral infections, including rotavirus, reovirus, severe acute respiratory syndrome coronavirus and HCV. The role of JNK and p38 signalling pathways during viral infection is to induce apoptosis or enhance viral replication (Clarke *et al.* 2004, Hassan *et al.* 2005, Kopecky-Bromberg *et al.* 2006, Holloway and Coulson 2006).

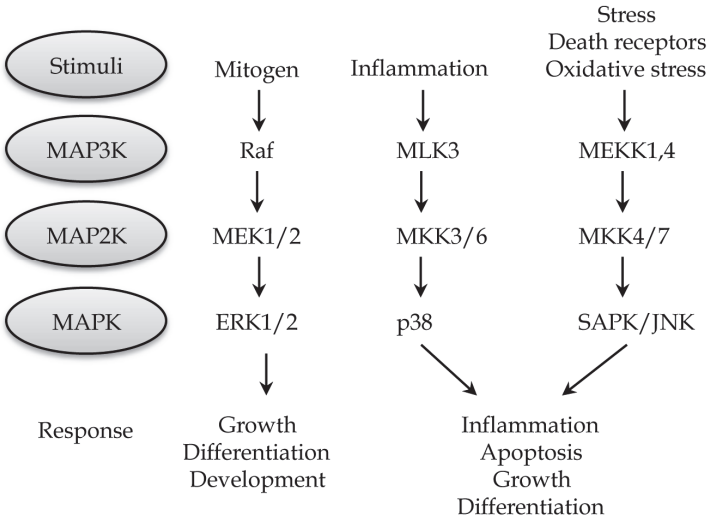

FIGURE 3 Representation of mitogen-activated protein kinase (MAPK) signalling cascades. MAPK signalling is activated by cell surface receptors. Figure demonstrates the three main MAPK pathways and examples of their activating stimuli and responses. ERK1/2, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; MLK, mixed-lineage kinase; MEKK, mitogen-activated protein kinase kinase kinase; MEK and MKK, mitogen-activated protein kinase kinase; SAPK, stress-activated protein kinase. Modified from Junttila *et al.* 2008

Calcium ion is an important signalling molecule. It is mainly stored in ER and additionally in the mitochondria (Clapham 2007). Mitochondria are in close contact with ER through the mitochondria-associated ER membranes (MAM). Important factors of the MAM are mitofusin proteins. Mitofusin 2 on the ER forms a complex with mitofusin 1 or 2 at the mitochondrial membrane that connects the ER to the mitochondria (de Brito and Scorrano 2008). MAMs enable direct calcium flux through this connection and hereby controls cell metabolism and death (Hayashi *et al.* 2009). Activation of calcium channels increases cytoplasmic calcium concentration and is followed by binding of calcium to certain calcium binding proteins, like calmodulin. Binding of calcium affects the shape and charge of that protein leading to the change in localization, association or function (Hoeflich and Ikura 2002, Clapham 2007). Mitochondria are involved in controlling the calcium concentration of the cytoplasm, but calcium also controls the mitochondrial function. Mitochondrial calcium concentration is involved in controlling of mitochondrial membrane potential and ATP production (Griffiths and Rutter 2009). Additionally, calcium signalling is involved in the mitochondrial pathway of apoptosis as high mitochondrial calcium concentration leads to permeabilization of mitochondrial membrane and release of proapoptotic molecules (Kroemer *et al.* 2007, Rasola and Bernardi 2007, Contreras *et al.* 2010). Disturbances in mitochondrial calcium concentration are frequently caused by viral infections. Increase in mitochondrial calcium

concentration and following apoptosis are detected e.g. during poliovirus and HCV infections (Piccoli *et al.* 2007, Brisac *et al.* 2010).

2.3.3 Viral infection restricts the progression of the cell cycle

As viruses are dependent on cellular functions for the synthesis of their macromolecules, they have evolved means to perturb normal functions of the cell. One such function is progression of cell cycle. Viruses interfere with the regulation of cell cycle for example to promote viral replication or to win time for viral assembly. Pathological consequences of viral regulation of cell cycle are connected to the cell death after arrest or induction of cancer. Premature and unscheduled transition to the following cell cycle phase creates cells with genomic instability and mutations leading to the formation of tumors (Gatza *et al.* 2005, Kannan *et al.* 2011).

A normal actively growing cell circles G_1 (gap 1), S (synthesis), G_2 (gap 2) and mitosis (M) phases of the cell cycle. Additionally, a resting cell can enter from the G_1 to G_0 phase (Fig. 4). During G_1 the biosynthetic activity of the cell is high as the cell is preparing RNA and proteins needed for DNA replication that occurs in the S phase. During replication, the DNA content of cells doubles from 2N to 4N. G_2 follows S phase and during G_2 cells synthesize the required macromolecules for the M phase when the nucleus and ultimately the whole cell divide to two daughter cells with 2N DNA content (Vermeulen *et al.* 2003). Viruses control the cell cycle to favor the phase important for the life cycle of that virus. For many viruses it means inducing S phase or preventing mitosis to keep cellular replication machinery active for viral needs (Chaurushiya and Weitzman 2009). The progression of cell cycle is regulated by cyclin-dependent kinases (CDK) that are activated by cyclin proteins (Fig. 4). Expression and degradation of different cyclins control the transition to the following phase of cell cycle. Additionally, CDK inhibitors control the activation of CDKs and the progression of the cell cycle. Before a cell enters to the following cell cycle phase it has to pass a checkpoint (Vermeulen *et al.* 2003, Branzei and Foiani 2008). Checkpoints control that all the steps of the replication process take place at the right phase of the cell cycle. If abnormalities are detected, the progression of cell cycle is halted to give time for the cells to recover back to their normal status (Branzei and Foiani 2008).

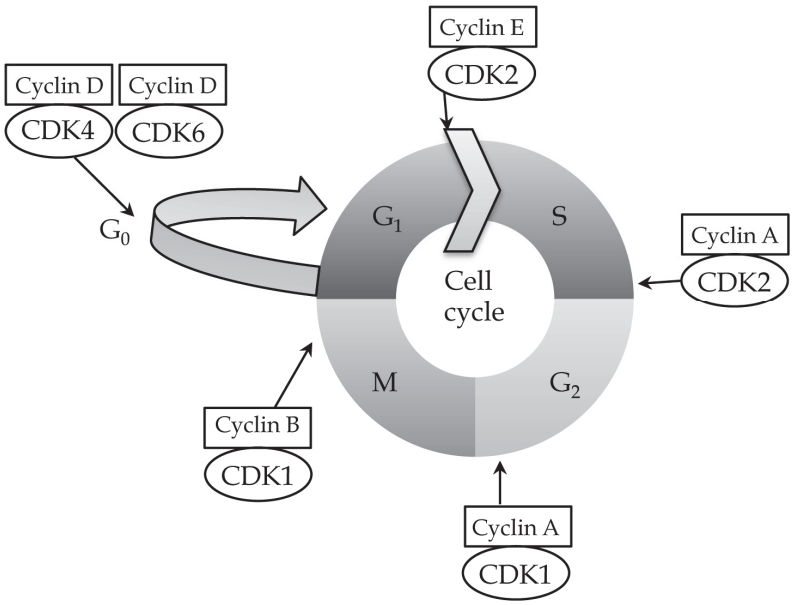

FIGURE 4 Regulation of the cell cycle by cyclin dependent kinases (CDK) and cyclins. Modified from Vermeulen *et al.* 2003.

Viruses interfere with checkpoints through their own proteins that mimic cyclins, by upregulating the expression of cellular cyclins, stabilizing cyclin-CDK complex or destabilizing CDK inhibitors (Gatza *et al.* 2005, Chaurushiya and Weitzman 2009). One example of viral regulation of CDKs is HCV. In HCV-infected cells, phosphorylation of CDK1 prevents the nuclear localization of cyclin B1 and infected cells accumulate in G_2 phase (Kannan *et al.* 2011). Influenza A virus induces a block at G_0/G_1 phase of cell cycle to support the production of progeny (He *et al.* 2010). A block is created by a decrease in Cyclins E and D1. Additionally, NS1 protein of influenza A virus interferes with Ras homolog gene family, member A (RhoA) signalling pathway inhibiting the expression of proteins regulating the transition from G_0/G_1 to S phase (Jiang *et al.* 2013).

Multiple cell cycle arrest mechanisms have been observed in parvovirus infected cells (Op De Beeck and Caillet-Fauquet 1997, Oleksiewicz and Alexandersen 1997). As a common feature, cell cycle is blocked so that infected cells do not enter mitosis (Morita *et al.* 2001). During B19V infection accumulation of cyclins A and B arrests cell cycle in G_2 phase. In addition, NS1 mediated G_1 arrest is observed. It is mediated by cooperation of NS1 with transcription factor Sp1 to activate CDK inhibitor p21 to block the cell cycle in G_1 (Morita *et al.* 2001, Morita *et al.* 2003, Nakashima *et al.* 2004). These studies have been conducted with an erythroid cell line semi-permissive to B19V infection. A more recent study with fully permissive erythroid progenitor cells described that NS1 interacts with E2F transcription factors to suppress their target gene expression

to establish a G_2 arrest during B19V infection (Wan *et al.* 2010). Lately, B19V induced cell cycle arrest in erythroid progenitor cells has been studied more in detail utilizing bromodeoxyuridine incorporation to detect DNA synthesis. Results from these studies demonstrated that the cell cycle is actually arrested at the S phase even though cells have a 4N DNA content (Luo *et al.* 2013). An S phase arrest has additionally been observed in non-permissive human liver carcinoma (HepG2) cells expressing the B19V NS1 protein (Kivovich *et al.* 2012). A MVM infection blocks the cell cycle at S and G_2 phases (Op De Beeck and Caillet-Fauquet 1997, Op De Beeck *et al.* 2001). The mechanism of cell cycle disturbances in MVM infection involves accumulation of p53 and its downstream effector CDK inhibitor p21. Furthermore, expression of NS1 is sufficient to induce cell cycle arrest suggesting a role for NS1 in cell cycle arrests (Op De Beeck and Caillet-Fauquet 1997). Disturbances in cell cycle frequently lead to cell death and contribute to pathology of parvovirus infection (Op De Beeck and Caillet-Fauquet 1997, Morita *et al.* 2001). The cell cycle progression of a CPV infection has not been studied yet.

2.3.4 Activation of DNA damage response in infected cells

The integrity of DNA is controlled strictly during cell cycle as DNA damage can induce developmental abnormalities and tumorigenesis. If DNA damage is detected, progression of cell cycle is blocked at checkpoint and DNA damage response (DDR) signalling is activated (Branzei and Foiani 2008). DDR pathway involves three PI3K-like kinases, ataxia telangiectasia-mutated kinase (ATM), ATM- and Rad3-related kinase (ATR) and DNA-dependent protein kinase (DNA-PK). ATM and DNA-PK are activated primarily as a result of DNA double-strand breaks while ATR responds to the detection of single-stranded DNA breaks and stalled DNA replication forks (Branzei and Foiani 2008, Giglia-Mari *et al.* 2011). Activated DDR kinases are recruited to the site of the DNA damage and they activate a cascade leading to the silencing of CDKs and cell cycle arrest allowing DNA repair or elimination of damaged cell through apoptosis (Branzei and Foiani 2008, Giglia-Mari *et al.* 2011).

Activation of DDR signalling has been reported during infections with DNA viruses like polyomaviruses, human papillomavirus (HPV), herpes simplex virus 1 (HSV-1) and HIV-1 (Roshal *et al.* 2003, Dahl *et al.* 2005, Lilley *et al.* 2005, Shi *et al.* 2005, Hein *et al.* 2009, Moody and Laimins 2009, Orba *et al.* 2010). DDR through ATM and ATR is activated by large T antigen of polyomaviruses Simian virus 40 (SV40) and John Cunningham virus (Shi *et al.* 2005, Hein *et al.* 2009, Orba *et al.* 2010). In HPV infected cells, viral proteins activate ATM pathway of DDR while formation of pre-replicative complexes initiates DDR in a HSV-1 infection (Lilley *et al.* 2005, Moody and Laimins 2009). Vpr protein of HIV-1 induces stalling of replication fork and activation of ATR signalling (Roshal *et al.* 2003, Zimmerman *et al.* 2006). During polyomavirus, HPV, HSV-1 and HIV-1 infections, activation of DDR leads to cell cycle arrest (Roshal *et al.* 2003, Lilley *et al.* 2005, Dahl *et al.* 2005, Moody and Laimins 2009, Orba *et al.* 2010). The block in cell cycle facilitates viral replication and production of progeny as inhibition of

DDR compromised viral replication (Dahl *et al.* 2005, Lilley *et al.* 2005, Moody and Laimins 2009, Orba *et al.* 2010). In contrast, EBV is able to attenuate a DDR induced by infection. As DDR is diminished, viral transformation of cells leads to a continuously proliferating B cell line and latent EBV infection (Nikitin *et al.* 2010).

MVM, B19V and MVC from autonomous parvoviruses have been reported to activate DDR (Adeyemi *et al.* 2010, Luo *et al.* 2011a, Luo *et al.* 2011b, Lou *et al.* 2012). Expression of viral proteins was not responsible for DDR, although the MVM NS1 was able to activate phosphorylation of H2A.X, a common marker of DNA damage and DDR (Rogakou *et al.* 2000, Adeyemi *et al.* 2010, Lou *et al.* 2012). Full DDR response was induced by viral replication and it is probably activated by viral replication intermediates or accumulation of strand brakes formed during replication (Adeyemi *et al.* 2010, Luo *et al.* 2011a, Lou *et al.* 2012). DDR signalling evidently has an important role in parvovirus infections as inhibition of DDR interferes with the viral replication and lowers production of progeny (Adeyemi *et al.* 2010, Luo *et al.* 2011a). DDR proteins accumulated in the site of MVM replication and it is possible that ATM directly enhances activity of NS1 by phosphorylation and hereby improves viral replication (Adeyemi *et al.* 2010).

The pathological consequence of viral interference with DDR depends on the strategy of viral action. DDR is important for productive infection of HSV-1 in epithelial cells inducing cold sores on lips (Lilley *et al.* 2005, Grinde 2013). Productive infection leads to death of host cells and transmission. From epithelial cells viruses spread to neurons where the infection is latent but can be reactivated by stimuli like UV light or stress and cause destruction of neuron (Grinde 2013). Latent infection of HSV-1 results from the inability of neurons to activate DDR (Lilley *et al.* 2005). Attenuation of DDR by EBV is followed by formation of immortalized B cell line (Nikitin *et al.* 2010). In contrast, activation of DDR contributes to oncogenic transformation of SV40 and HPV infections (Moody and Laimins 2009, Hein *et al.* 2009). During HIV-1 infection DDR signalling is connected to the arrest of cell cycle and subsequent apoptosis of $CD4^+$ cells (Roshal *et al.* 2003). Correspondingly, given the role in facilitating parvoviral replication, the activation of DDR accounts for the cytotoxic outcome and the pathology of parvovirus infection (Adeyemi *et al.* 2010, Luo *et al.* 2011a). As DDR signalling has a role in viral replication and production of progeny it could be targeted with drugs to treat viral infections (Moody and Laimins 2009, Luo *et al.* 2011b).

2.3.5 Induction of cell death by viral proteins

Cell death pathways are important processes in normal development and maintenance of tissue homeostasis. However, when cell death pathways are disregulated they contribute to various diseases (Levine and Kroemer 2008, Agostini *et al.* 2011). Cell pathogens can also take over the control of cell death pathways leading to progression to diseases (Abdel-Latif *et al.* 2006, Nakashima *et al.* 2006, Cummins and Badley 2010, Sir and Ou 2010, Ashida *et al.* 2011, Quarato *et al.* 2013). A cell can die through different pathways, most of which are

controlled (Kroemer *et al.* 2009). The three main pathways for cell death are apoptosis, autophagy and necrosis (Table 2). However, there are numerous occasions when dying cell displays features of more than one cell death type and the classification of the cell death may be artificial. In this section autophagy and necrosis will be first described shortly and then the apoptotic pathway will be discussed more in detail together with few examples of cell death pathways induced by viral infections.

2.3.5.1 Autophagy

Autophagy is a catabolic pathway involved in degradation of cytoplasmic material. Autophagosomes are double-membraned vesicles that first engulf cytoplasmic material or even organelles and then fuse with endosomes and lysosomes to form a degradative vesicle. Contents of the vesicle are degraded and released to the cytoplasm for reuse. Indeed, autophagy is a survival mechanism during short-term starvation. Autophagy is also involved in development and growth regulation (Edinger and Thompson 2004, Eskelinen 2005). In cell death by autophagy, autophagic vesicles accumulate in the cytoplasm and the cell digests itself (Kroemer and Levine 2008). Autophagic cell is characterized by accumulation of double membrane vesicles and absence of chromatin condensation (Table 2). Genes controlling autophagy are named Atg (autophagy related). Atg8 (or microtubule associated protein 1 light chain 3, LC3) and Atg6 (beclin 1) are involved in the formation of autophagic vesicles and are widely used as a marker of autophagocytosis (Kroemer *et al.* 2009). Some viruses, like HSV-1, have means to suppress autophagy since it has a role in innate immunity to destroy intracellular pathogens. Thus suppression of autophagy enhances virulence and pathogenesis. Viruses may additionally exploit autophagy. RNA viruses, like HCV, HIV-1 and poliovirus, utilize autophagosomes in their RNA replication. In addition, autophagy is exploited in the release of progeny of poliovirus and HIV-1. Viral induction of autophagy may result is cell death as in adenovirus infection or it may be beneficial for virus as SV40 virus induces autophagocytosis to ensure energy supply (Sir and Ou 2010). From parvoviruses, activation of autophagy has been observed during B19V infection. Presumably autophagy has a role in the survival of infected cells and it ensures replication of B19V (Nakashima *et al.* 2006). Otherwise activation or suppression of autophagy during parvovirus infection has not been reported.

TABLE 2 Discrimination of apoptosis, autophagy and necrosis according to their morphological and biochemical features. Modified from Kroemer *et al.* 2009, Rogakou *et al.* 2000.

Cell death type	Morphological features	Biochemical features
Apoptosis	Rounding up	Activation of proapoptotic Bcl-2 family proteins
	Decrease of cellular and nuclear volume	Mitochondrial membrane depolarization
	Nuclear fragmentation	Activation of caspases
	Minor modification of cell organelles	DNA damage, histone H2A.X phosphorylation
	Plasma membrane blebbing	Phosphatidylserine exposure
		ROS overproduction
Autophagy	Absence of chromatin condensation	Beclin-1 dissociation from Bcl-2
	Vacuolization of the cytoplasm	Conversion of LC3-I to LC3-II
	Vesicles surrounded by double membrane	
Necrosis	Increase in cellular volume	Activation of calpains and cathepsins
	Rupture of plasma membrane	Drop in ATP lelvel
	Swelling of cell organelles	Lysosomal membrane rupture
	Minor chromatin condensation	Plasma membrane rupture
		ROS overproduction

2.3.5.2 Necrosis

Necrosis has been considered as an accidental and unprogrammed cell death type. However, it has recently been proposed that the occurrence of necrosis may be a regulated event as it can be triggered by specific ligand/receptor binding. In addition, the progression of necrosis may be controlled and certain intracellular events are often observed in necrotic cells (Table 2). These include early rupture of plasma membrane, production of reactive oxygen species (ROS), activation of calpains and cathepsins and increase in cytoplasmic calcium concentration. Together with apoptosis, necrosis is involved in normal development and tissue homeostasis. Necrosis may also act as a backup cell death mechanism when a dying cell fails to go through apoptosis or autophagy (Golstein and Kroemer 2007, Kroemer *et al.* 2009). Similar to autophagy, necrosis is utilized by viruses to induce death of host cell. From parvoviruses, BPV has been reported to induce necrosis that accounts for release of progeny (Abdel-Latif *et al.* 2006). Necrosis frequently induces inflammation and immune response towards necrosis-inducing agent. This is observed in vaccinia virus infected mouse where infection induces necrosis that reduces the production of new viruses and decreases the

severity of infection (Cho *et al.* 2009). To prevent inactivation of virus by immune response, viruses, like murine cytomegalovirus, encode proteins to inhibit necrosis and ensure viral replication (Upton *et al.* 2012).

2.3.5.3 Apoptosis

Commonly, apoptosis is defined as a programmed cell death including morphological changes like rounding of the cell, chromatin condensation, nuclear fragmentation and plasma membrane blebbing (Table 2). Apoptosis can proceed through extrinsic pathway activated by extracellular ligand binding to certain receptors on plasma membrane or intrinsic pathway initiated by intracellular signalling (Fig. 5) (Kroemer *et al.* 2009). Multiple cell death pathways are controlled by the mitochondria, as they are able to sense cellular stress through signalling networks and control responses to these signals (Green and Reed 1998, Galluzzi *et al.* 2012). Response can be adaptive or signal for death depending on the level of the danger. In a same way mitochondria also signal the cell about perturbations in mitochondrial homeostasis (Galluzzi *et al.* 2012). Mitochondria are important mediators especially in the intrinsic apoptotic pathway. This pathway involves permeability transition of mitochondrial inner membrane that leads to equilibration of ions between the mitochondrial matrix and intermembrane space, uncoupling the respiratory chain and loss of the mitochondrial membrane potential. These events are followed by the mitochondrial release of proteins involved in apoptosis and set the point of no return for cell death (Zamzami *et al.* 1995a, Green and Reed 1998, Green and Kroemer 2004, Galluzzi *et al.* 2012).

Caspases are cysteine proteases that mediate cell death through both pathways of apoptosis (Fig. 5) (Kumar 2007). Initiator caspases are activated in the early phases of apoptotic pathway and once active they activate effector caspases. Effector caspases cleave cellular substrates and cause final disassembly of the cell (Taylor *et al.* 2008). Initiator caspases are caspses 2, 8, 9 and 10. Caspase 3 is the main effector caspase but also effector caspases 6 and 7 exist (Kumar 2007). Effector caspases, among other substrates, damage cell junctions, reorganize cytoskeleton, stop DNA replication and repair, disrupt nuclear structure, induce phagocytosis and finally disintegrate cell into apoptotic bodies (Taylor *et al.* 2008). *In vivo* apoptotic bodies are engulfed by surrounding cells to prevent the complications caused by the release of intracellular contents (Thornberry and Lazebnik 1998). When studying the activation of caspases one has to bear in mind that caspases function also in other cellular situations not connected to cell death, like nuclear factor κB activation, cell proliferation, differentiation and motility (Lamkanfi *et al.* 2007).

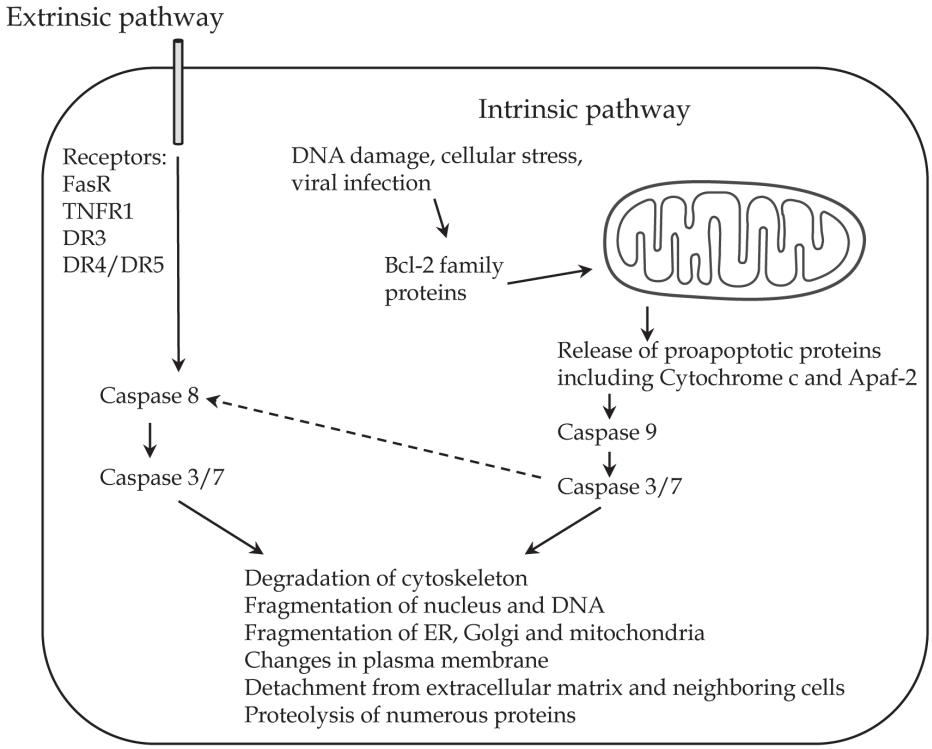

FIGURE 5 Simplified representation of extrinsic and intrinsic apoptotic pathways. Extrinsic pathway is activated by binding of ligand to the death receptor at cell surface leading to the activation of caspase 8. Intrinsic pathway is initiated by intracellular stimuli and mediated by mitochondria to activate caspase 9. Apaf-2, apoptotic protease activating factor 2; Bcl-2, B cell lymphoma 2; TNFR1, tumor necrosis factor receptor 1; DR, death receptor. Modified from Galluzzi *et al.* 2008 and Taylor *et al.* 2008

Intrinsic apoptotic pathway proceeds through signalling via B cell lymphoma 2 (Bcl) family proteins (Fig. 5). Death signal, e.g. DNA damage, leads to cytoplasmic release of cytochrome c and apoptotic protease activating factor 2 (Apaf-2) from mitochondria (Kumar 2007). Additionally dissipation of mitochondrial membrane potential and production of ROS have been connected to the release of cytochrome c (Simon *et al.* 2000, Gottlieb *et al.* 2003). In the cytoplasm cytochrome c and Apaf-2 with procaspase 9 form an apoptosome where caspase 9 is activated. Caspase 9 then activates effector caspases to accomplish cell death (Kumar 2007).

Extrinsic apoptosis is initiated by binding of a ligand to death receptor on cell membrane (Fig. 5). Two main death receptors are Fas and tumor necrosis factor (TNF) receptor. Upon ligand binding to receptor the signal is transmitted to cytoplasm and culminates in activation of caspase 8 (Ashkenazi and Dixit 1998, Kumar 2007). Execution of apoptosis is then finalized through activation of effector caspases (Kumar 2007).

2.3.5.4 Viral mechanism to induce cell death

Numerous viral proteins induce apoptosis in infected host cell. Apoptosis accounts for tissue injury of viral diseases and is considered as a pathogenic mechanism of viral diseases (Galluzzi *et al.* 2008, Clarke and Tyler 2009). In addition to several viral proteins that have been reported to induce cell death, viral RNA and DNA may signal for apoptosis (Hirsch *et al.* 2011, Liu *et al.* 2014). Two examples of viral protein induced cell death pathways and their role in pathogenesis are described below.

HIV-1 infection involves loss of $CD4^+$ (CD, cluster of differentiation) T lymphocytes and a weakened immunity leads to the acquired immunodeficiency syndrome. The loss of $CD4^+$ T lymphocytes is produced by HIV-1 induced apoptosis. Many of the HIV-1 proteins have proapoptotic characteristics accounting to the death of the host cells. Both the extrinsic and intrinsic pathways are utilized by HIV-1 proteins in inducing apoptosis (Cummins and Badley 2010). HIV-1 accessory protein viral protein R (Vpr) is largely responsible for the pathogenesis of HIV-1 because of its ability to induce cell cycle arrest as well as apoptosis. Apoptosis induced by Vpr protein is mediated by mitochondria. Vpr is transported from the ER through MAM to the mitochondrial outer membrane. Integration of Vpr to the mitochondrial membrane disrupts it leading to the depolarization of mitochondrial membrane potential. Depolarization is additionally mediated by Vpr-related reduction in expression of mitofusin 2, a protein involved in normal mitochondrial dynamics (Jacotot *et al.* 2000, Huang *et al.* 2012). Mitochondrial fusion and fission contribute to the maintaining of functional mitochondria and decrease the effect of damaged mitochondria as they are mixed with intact ones (Youle and van der Bliek 2012). Mitochondrial disturbances together with a defect in mitochondrial dynamics lead to apoptotic cell death as cytochrome c is released from mitochondria and caspases are activated (Jacotot *et al.* 2000, Huang *et al.* 2012). In addition, HIV-1 envelope glycoprotein gp160 activates mitochondrial apoptotic pathway by increasing cytoplasmic calcium and inducing damage to DNA (Sasaki *et al.* 1996, Sasaki *et al.* 2002). On the other hand, another HIV-1 envelope glycoprotein 120 has been reported to induce apoptosis through extrinsic pathway. Tat, the transactivator of HIV-1, affects the expression of apoptosis related proteins and thus induces apoptosis. Interestingly, many of HIV-1 proteins have additionally antiapoptotic properties (Cummins and Badley 2010).

HCV is a RNA virus causing chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Replication and protein synthesis of HCV take place in ER and creates an unfolded protein ER stress response (Moradpour *et al.* 2007, Quarato *et al.* 2013). ER stress induces release of calcium to cytoplasm (Gong *et al.* 2001, Quarato *et al.* 2013). Mitochondria are involved in buffering the cytoplasmic calcium concentration and calcium released from ER is taken into mitochondria. Increased mitochondrial calcium leads to production of ROS, permeabilization of mitochondrial membrane, disruption of mitochondrial membrane potential and release of proapoptotic proteins. Additionally mitochondrial calcium uptake is increased by HCV proteins that have been reported to localize to the

mitochondria (Korenaga *et al.* 2005, Piccoli *et al.* 2007). The established oxidative stress inhibits mitochondrial respiratory complex I and further enhances ROS production. However, despite of the mitochondrial disturbances, increased production of ATP is observed in HCV-infected cells. This is achieved by a compensatory shift in metabolism to glycolysis (Piccoli *et al*. 2007). Disruptions in mitochondrial metabolism lead to accumulation of metabolites that activates the transcription factor hypoxia-inducible factor 1 (HIF-1) that controls the expression of glycolytic enzymes. In addition, HIF-1 controls expression of antiapoptotic factors providing prosurvival signalling (Ripoli *et al.* 2009). These diverse mechanisms regulate the different modes of HCV pathology. The level of alterations in calcium and ROS homeostasis account for the observed pathology. When homeostasis is only slightly affected a persistent infection involving HIF-1 activation of antiapoptotic protein is established. However, additional factors may cause persistent HCV infection to lead to hepatocarcinoma. If change in calcium and ROS homeostasis is more profound also metabolic changes are more profound. This causes accumulation of lipid droplets and steatosis. Tissue fibrosis is caused by HCV when high mitochondrial calcium and ROS concentration is induced. Then mitochondrial membrane is permeabilized and proapoptotic proteins are released to cytoplasm. Depending on the energy status of the cell this leads either to apoptosis or necrosis (Quarato *et al.* 2013).

2.3.5.5 Mechanisms of cell death induced by parvoviruses

Cytotoxic effects caused by different parvoviruses differ, depending on the infecting virus and also the cell type. Parvoviral infection or expression of parvoviral proteins have been reported to induce apoptosis, necrosis and also other mechanisms for cell death have been suggested (Abdel-Latif *et al.* 2006, Nuesch and Rommelaere 2007, Chen and Qiu 2010). Virus induced cell death has a direct link to the observed pathology of disease (Macartney *et al.* 1984, Anderson *et al.* 1985, Ohshima *et al.* 1998).

B19V infection induces apoptosis in infected cells but the mechanism of apoptosis depends on the infected cell line. In non-permissive hepatocytes the B19V infection and NS1 expression induce intrinsic apoptotic pathway involving DNA damage and activation of caspases 3 and 9 (Poole *et al.* 2004, Poole *et al.* 2006, Poole *et al.* 2011). In semi-permissive erythroid progenitor cells NS1 protein induced an extrinsic apoptotic pathway including activation of caspases 3, 6 and 8 probably through tumor necrosis factor α pathway (Moffatt *et al.* 1998, Sol *et al.* 1999). However, a study with a fully permissive primary erythroid progenitor cells demonstrated that the small 11 kDa NS protein was the main inducer of apoptosis during B19V infection and this apoptosis involved activation of caspase 10 suggesting extrinsic apoptotic pathway (Chen *et al.* 2010). Also infections of CPV and FPV or expression of CPV NS1 induce apoptotic cell death as observed *in vitro* and *in vivo* (Ikeda *et al.* 1998, Bauder *et al.* 2000, Saxena *et al.* 2013), but the mechanism of apoptosis has not been studied. In addition, ADV infection induces activation of caspase 3 and apoptotic cell death occurs. Interestingly, caspase 3 has a role in ADV life cycle as cleavage of NS1 protein by

caspase 3 was found to be a prerequisite for ADV replication (Best *et al.* 2002, Best *et al.* 2003).

In contrast to the clear apoptotic cell death involved in B19V, CPV, FPV and ADV infections, the cell death type involved in H-1PV and MVM infections are more divergent. MVM infection has been reported to induce cell lysis in permissive murine cells (Cotmore and Tattersall 1987). The morphology of MVM infected cells is changed during infection and induction of cytopathic effect has been shown to involve a NS1 mediated interference in the regulation of cytoskeleton dynamics leading to rearrangement and degradation of micro and intermediate filaments (Corbau *et al.* 2000, Nuesch *et al.* 2005). Induction of morphological changes involves linking of tropomyosin to casein kinase II through NS1. This results in altered activity of the kinase causing cell disturbances and lysis (Nuesch and Rommelaere 2006, Nuesch and Rommelaere 2007).

H-1PV induces apoptosis in infected rats and in rat cell culture (Ohshima *et al.* 1998). Apoptosis is also observed in H-1PV infected human cell line mediated by NS1 protein. The apoptotic pathway involves TNFα or accumulation of reactive oxygen species and DNA damage depending on the cell line (Rayet *et al.* 1998, Hristov *et al.* 2010). However, in apoptosis resistant cells H-1PV infection induces cytoplasmic accumulation of lysosomal cathepsins B and L and the observed cell death is necrosis (Di Piazza *et al.* 2007). Necrosis is additionally induced in BPV infected embryonic bovine tracheal cells (Abdel-Latif *et al.* 2006).

2.3.6 Cytoskeletal rearrangements

Cell shape, motility, endocytosis and intercellular communication are among cellular functions dependent on cytoskeleton formed of actin, intermediate filaments and microtubulin. Viruses exploit and induce alterations in cytoskeleton during different states of their life cycle including entry and spread of progeny (Suikkanen *et al.* 2003b, Nuesch *et al.* 2005, Bär *et al.* 2008, Taylor *et al.* 2011, Sobo *et al.* 2012). Binding of echovirus 11 to the cell surface induces rapid reorganization of actin filaments essential for viral entry. Additionally, later during echovirus 11 infection the actin filaments are degraded probably due viral protein synthesis (Sobo *et al.* 2012). Parvoviruses utilize microtubules during entry and egress (Suikkanen *et al.* 2003b, Bär *et al.* 2008) while actin is remodeled late in infection (Nuesch *et al.* 2005). In addition to the use of cytoskeleton during viral entry and egress, replication of some viruses is enhanced by actin (Harpen *et al.* 2009). Cytoskeleton is also utilized in transport of viral genomes and proteins to the site of assembly. Rearrangements and degradation of cytoskeleton during viral infection are associated with cell death, tumorigenesis and viral spread (Taylor *et al.* 2011). Rous sarcoma virus infections induce multiple changes in cytoskeletal proteins affecting cell shape and inducing invasive phenotype with loss of contact inhibition. These changes are connected to tumorigenesis of viral infection. Similar host cell transformation causing tumorigenesis occurs during SV40 and hepatitis B virus infections (Taylor *et al.* 2011).

Various viruses induce formation of protrusions at the late stage of infection to be utilized in viral spread. These include coxsackievirus B3, alphaherperviruses, Marburgvirus and retroviruses. Protrusions contain either actin or microtubulin of both of these filaments (Favoreel *et al.* 2005, Sherer *et al.* 2007, Kolesnikova *et al.* 2007, Paloheimo *et al.* 2011, Nikolic *et al.* 2011). Protrusions are formed after viral proteins activate cellular signalling cascades to induce rearrangement of cytoskeleton (Favoreel *et al.* 2005, Nikolic *et al.* 2011). Protrusions form intercellular connections from infected cell to uninfected ones. Studies have revealed that protrusions are utilized in direct cell-to-cell transport of newly made viruses (Favoreel *et al.* 2005, Sherer *et al.* 2007, Kolesnikova *et al.* 2007, Nikolic *et al.* 2011, Paloheimo *et al.* 2011). Protrusions facilitate effective way for viral spread as viruses are hidden from immune system. Intercellular connections additionally offer a non-lytic spread for non-enveloped viruses alongside lytic infections (Paloheimo *et al.* 2011). Parvoviruses have been suggested to use a regulated egress pathway involving vesicular transport along microtubules (Bär *et al.* 2008, Bär *et al.* 2013) but the possible direct cell-to-cell transport of progeny has not been studied.

3 AIMS OF THE STUDY

Viruses have evolved to be able to replicate in host cells despite cellular defence mechanisms. Viruses have means to hide from cells or antagonize cellular anti-viral pathways. Understanding the mechanisms of viral pathology is crucial for development of new viral therapies and design of new antiviral drugs. In addition, divergent mechanisms of virus-host interactions have to be taken into account when viruses are utilized as tools in medicine. Mechanism of parvovirus pathology has been studied to an extent and common features have been found. However, the early response to infection and the role of mitochondria and mitochondria-virus interaction has not been studied in parvovirus infections. The aims of this doctoral thesis were:

1. To study the time course of host cell response to a CPV infection.
2. To characterize the mechanism of cytopathy and cell death response.
3. To analyze cellular morphological changes caused by an infection.

4 SUMMARY OF THE MATERIALS AND METHODS

The materials and methods used in these studies are listed in Table 3. Detailed descriptions can be found in the original papers indicated with Roman numerals.

TABLE 3 Summary of the materials and methods used in the thesis. The Roman numerals refer to the original articles where the detailed descriptions can be found.

Material/Method	Publication
NLFK/A72 cell culture	I, II, III
CPV-2 purification and infection of cells	I, II, III
Cell synchronization with thymidine	II
DIC microscopy	I, III
Confocal microscopy	I, II, III
Electron microscopy	I, II
Flow cytometry	I, II
Cell cycle analysis	I
Membrane potential analysis	I
Mitochondrial membrane potential analysis	I, II
Annexin V/PI assay	I
Caspase activity assay	I
Immunofluorecence labelling	I, II, III
DNA fragmentation	I
Colocalization analysis	II
Analysis of cytoplasmic calcium concentration	II
Western blotting	II
ERK1/2 activation/inhibition	II

5 REVIEW OF THE RESULTS

5.1 General course of CPV infection

5.1.1 Time scale of CPV replication

As NS1 protein is expressed shortly after beginning of viral replication (Cotmore and Tattersall 1987), expression of this protein was examined to establish a time scale for CPV infection. CPV infected cells were labelled with monoclonal antibody towards NS1 protein and immunofluorescence microscopy was utilized to determine the percentage of cells expressing NS1. Results demonstrated that in Norden laboratory feline kidney (NLFK) cells at 10 h post infection (p.i.) 6.4% of the cells were expressing viral NS1 protein indicating onset of viral replication (I, Fig. 5A). Amount of cells expressing NS1 increased gradually so that at 24 h p.i. 91.0% of the cells were positive for NS1. In A72 cells the infection proceeded slower and at 24 h p.i. only few cells were expressing NS1 (I, data not shown). At 48 h p.i. percentage of A72 cells positive for NS1 had reached to 58%.

5.1.2 Time scale of cytopathy in CPV infected cells

In order to obtain a general understanding of the cytopathic effect of CPV infection, differential interference contrast (DIC) microscopy was used. This kind of light microscopic method can be utilized to detect the global morphological changes associated with later phases of cell death (Galluzzi *et al.* 2009). NLFK and A72 cells were infected with CPV and visualized as unfixed samples (I, Fig. 1). As previous results of NS1 expression revealed that infection proceeded slower in A72 cells than in NLFK cells, different time points were chosen for different cell lines. In CPV infected NLFK cells no changes in cell morphology were observed at 12 h p.i. (I, Fig. 1A) when viral replication is starting. Some rounded cells were observed at 24 h p.i., but at 48 h p.i. majority of the cells had detached or had morphological changes as cells were shrunken, rounded and had long protrusions. At 72 h p.i. protrusions were longer and more numerous (III, Fig. 1E). Accordingly, no morphological changes were detected at 24 h p.i. in

infected A72 cells (I, Fig. 1B). However, at 48 h p.i. rounding and detaching of the cells were observed and at 72 h p.i. only a minority of the cells remained at the culture. Long extensions were observed also in CPV infected A72 cells at 48 and 72 h p.i.. According to these results the time points between beginning of NS1 expression and immense cell detaching were chosen for both cell lines to study further the mechanism of CPV induced cytopathy.

5.2 CPV infection arrests progression of cell cycle

Visual examination of CPV infected cells with DIC microscopy (I, Fig. 1) indicated that the amount of cells in CPV infected samples declined when compared to mock infected sample. This indicated halting of cell division and was further examined. Ethanol fixed cells were incubated with propidium iodide (PI) to label the DNA and flow cytometric analysis of DNA content of CPV infected cells were conducted to investigate the progression of cell cycle. In CPV infected NLFK cells accumulation of cells in S phase was observed (I, Fig. 2A). Already at 12 h p.i. the proportion of cells in G_0/G_1 phase had declined and accordingly proportion of cells in S phase had increased from 19.3% to 30.4% when compared to mock infected cells. At 24 h p.i. most of the cells were in S phase (50.2%) or had more DNA than in normal G_2 phase (>G_2/M, 19.8%). The extra DNA detected in infected cells accounts for the accumulation of viral DNA in the cell. Similar cell cycle arrest was observed in CPV infected A72 cells (I, Fig. 2B). Arrest was established later and with the exception that at 72 h p.i. majority of the cells, 36.0%, were in the group of cells having more DNA than at normal G_2/M phase while 16.7% of the cells were in S phase. These results suggested that cell cycle was blocked at S phase in CPV infected cells and as cell division was not occurring cells accumulated unusually high amounts of DNA.

5.3 Identification of the mechanism of CPV induced cytopathy

5.3.1 CPV infection affects the integrity of plasma membrane

Translocation of phosphatidylserine from the inner leaflet to the outer leaflet of plasma membrane is considered as a marker of apoptotic cell death (Martin *et al.* 1995). Externalization of phosphatidylserine can be examined with Annexin V labelling. Furthermore, PI is a dye staining DNA but PI is not able to cross intact plasma membrane seen in living cells. When double labelling with Annexin V and PI is analysed with flow cytometry cells can be distinguished to be intact (no staining), apoptotic (Annexin V positive) or necrotic (PI positive). Staurosporine (STS), an antibiotic commonly used to induce apoptosis, was utilized as a positive control for apoptosis. When NLFK cells were incubated with STS, a group of Annexin V positive and PI negative cells were detected indicating these

cells to be apoptotic (I, Fig. 3A, sts). Mock-infected cells were negative for both dyes (I, Fig. 3A). When cells were infected with CPV, predominantly the amount of cells positive for both, Annexin V and PI, dyes were increasing indicating changes in plasma membrane, specific to necrosis. At 12 h p.i. the amount of double positive cells had increased from 1.9% for mock infected cells to 3.5% for CPV infected cells. Furthermore, at 23 h p.i. 50.6% of CPV infected cells were double positive demonstrating necrosis. However, increase of apoptotic cells was detected at 23 h p.i. when 22.9% of cells were positive only for Annexin V.

With CPV infected A72 cells corresponding phenomenon was detected (I, Fig. 3B). Amount of double positive cells increased after infection but also the amount of apoptotic cells increased. This was seen especially at 48 h p.i. when 10% increase in the amount of both apoptotic and double positive cells was seen when compared to mock infected cells. At 72 h p.i. the amount of apoptotic cells decreased and the amount of double positive cells increased accordingly. Taken together, these results demonstrated the apoptotic translocation of phosphatidylserine, but also more profound changes at the plasma membrane suggesting necrosis.

5.3.2 Involvement of caspases in CPV cytopathy

As Annexin V and PI labelling did not reveal the classic apoptotic labelling, mechanism of cell death was further investigated. Caspases are proteases involved especially in apoptotic cell death pathway (Kumar 2007). Activity of caspases 3/7, 8 and 9 was examined in CPV infected cells. STS was used as a positive control to activate caspases and results were normalized to STS treated cells. CPV and mock-infected cells were compared to STS treated cells. Already at 6 h p.i. significant activation of caspase 9 was detected in CPV infected NLFK cells (I, Fig. 4A). At that timepoint also caspase 8 was activated to some extent. The activity of caspases 8 and 9 increased to 12 h p.i. but then started to decline being still statistically significant. Activity of caspase 3/7 was detected to increase gradually from 12 h p.i. to 48 h p.i.. At 24 h p.i. activity of caspase 3/7 was statistically significant, but it did not reach the same level than that of caspases 8 and 9. At 48 h p.i. also the caspase 9 was still active but the activity of caspase 8 had declined to the level of mock infected cells.

In CPV infected A72 cells statistically significant activation of caspases 3/7 and 9 was detected from 12 h p.i. to 72 h p.i. (I, Fig. 4B). Caspase 8 was activated at 24 h p.i. but at 48 h p.i. activity has diminished. These results are in accordance to the function of different caspases, for caspase 9 and 8 being initiator caspases activating caspase 3/7 that then acts as an executioner caspase. Activation of caspases during CPV infection indicates apoptotic pathway for CPV induced cell death.

5.4 CPV infection induces damage to the cellular DNA

As above results indicated onset of apoptotic cell death pathway, integrity of nuclear DNA was studied in order to find the initiating signal for apoptosis. Fragmentation of DNA and phosphorylation of histone H2A.X, a marker of DNA damage (Rogakou *et al.* 2000), are hallmarks of apoptosis. These two markers were examined in order to study the cell death mechanism of CPV infected NLFK cells. Phosphorylation of H2A.X was tested with immunofluorescence labelling of cells (I, Fig. 5A). At the same time expression of viral NS1 protein was detected with NS1 antibody. Percentage of cells showing fluorescence from phospho–H2A.X or NS1 was calculated. At 8 h p.i. cells started to express NS1 protein and at the same time increase in phospho–H2A.X was seen. Increase in NS1 positive cells was accompanied by accumulation of phospho–H2A.X indicating DNA damage and apoptosis.

Electron microscopy of CPV infected NLFK cells revealed condensation of DNA at the nuclear envelope at 24 h p.i. (I, Fig. 5B). At 48 h p.i. blebbing of nuclear membrane was seen. Agarose gel electrophoresis of DNA from CPV infected NLFK cells demonstrated fragmentation of DNA at 24 and 48 h p.i. (I, Fig. 5C) supporting apoptotic type of cell death.

5.5 Interaction of CPV with mitochondria

5.5.1 CPV infection induces depolarization of mitochondrial membrane potential

To define the role of mitochondria in CPV induced pathology, the membrane potential of mitochondria was examined. 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide as a trademarks DePsipher (I) or JC-1 (II) was used to detect the mitochondrial membrane potential. Accumulation of this dye to mitochondria is dependent on mitochondrial potential. In depolarized mitochondria the accumulation of the dye is low and the dye is as monomers that have green fluorescence. In polarized mitochondria the accumulation of the dye is higher and dye monomers form an aggregate that has a red fluorescence. K^+-selective ionophore valinomycin disrupting the mitochondrial membrane potential was used to create a control sample with depolarized mitochondria. Mock and CPV infected cells were compared to valinomycin treated cells to detect the depolarization of mitochondrial membrane potential. Biphasic depolarization of mitochondrial membrane potential was discovered. Initial depolarization of mitochondrial membrane was observed at 2 h p.i. (II, Fig. 3). However, the potential was restored at 4 h p.i.. Then later in infection, starting at 14 h p.i., mitochondria were detected to be depolarized. Amount of cells having depolarized mitochondria increased during infection so that at 24 h p.i. 79.5% of CPV infected cells had depolarized

mitochondria (I, Fig. 6B). These results proposed that cellular survival signalling was activated in the beginning of CPV infection as mitochondrial membrane potential was restored. At later time points the survival signalling was overridden and mitochondrial membrane potential was diminished.

5.5.2 Association of CPV with mitochondria

The observed depolarization of mitochondrial membrane potential and activation of caspase 9 prompted us to study the association of CPV with mitochondria in order to explore whether the association of CPV with mitochondria was enough to induce cell death in infected cells. Immunofluorescence studies and colocalization analysis were performed. Mitochondria were labelled with MitoTracker Red (MTR), a dye that accumulates in active mitochondria, or with antibody towards mitochondrial inner membrane protein cytochrome c oxidase IV (COX IV). CPV was labelled with anti-capsid antibody recognizing intact capsid. Confocal microscopy images (II, Fig. 1A) revealed that CPV localized close to mitochondria in all studied time points 2 – 24 h p.i.. Colocalization analysis was conducted with BioImage XD software to analyse localization of CPV in more detail. Percentage of colocalization between CPV and mitochondria labelled with MTR was already 13% at 2 h p.i.. Then the percentage declined and at 10 h p.i. the amount of colocalization started again to increase. The maximum colocalization was detected at 24 h p.i.. Almost similar pattern was seen with anti-COX IV labelled mitochondria. Colocalization was low during the early phases of infection but colocalization percentage increased at later phases of infection starting from 16 h p.i.. These results demonstrated close association of CPV with mitochondria.

5.5.3 Damage to mitochondria

Due to the observed colocalization with confocal microscope, transmission electron microscopy was used to study more in detail the localization of CPV with respect to the mitochondria. Also ultrastructure of mitochondria from infected cells were studied. Nanogold labelling with silver enhancement was utilized to detect CPV. Immunogold labelling studies revealed that the virus localized at the mitochondrial membrane, but only occasionally inside the mitochondria (II, Fig. 2A). All the mitochondria from one CPV infected cell were examined and analyzed as intact or damaged. Damaged mitochondria had deteriorations on membrane, as holes or membrane blebbing, or disappearance of cristae. All together 30 cell per time point were analyzed and results show that amount of damaged mitochondria increased already at 2 h p.i. when 24% of mitochondria were damaged (II, Fig. 2B). At 6 h p.i. amount of damaged mitochondria decreased but at 10 h p.i. mitochondria were again harmed in CPV infected cells. After 10 h p.i. percentage of damaged mitochondria increased so that at 18 h p.i. it was 46% and then it decreased to 40% by 22 h p.i.. Decrease in percentage of damaged mitochondria at 18 – 22 h p.i. was due to increase in cell death and cells were easily detaching during sample preparation. Electron

microscopy studies revealed that CPV was not present inside mitochondria but associated with outer mitochondrial membrane. Results also suggested that CPV infection induced damage to mitochondrial structure as damage to mitochondrial outer membrane was observed.

5.6 Influence of CPV infection in cell signalling molecules

5.6.1 Reactive oxygen species

Increased production of ROS is often associated with damage to proteins, lipids, DNA and mitochondria (Sinha *et al.* 2013). Production of ROS was measured from CPV infected cells from 2 h p.i. to 24 h p.i.. Additionally, the ROS level was quantified from mock infected cells, from apoptotic cells (apoptosis induced with staurosporine), and from cells incubated with a ROS-inducing compound tert-butyl hydroperoxide. The production of ROS increased already at 2 h p.i. when the ROS level was 1.4 times the level in mock infected cells (II, Fig. 4). The highest ROS level was detected at 6 h p.i. when it was 2.1 times the level in mock infected cells. Thereafter the ROS level returned to the level of mock infected cells but started to increase again at 24 h p.i.. These results demonstrated induction of oxidative stress during CPV entry.

5.6.2 Calcium

Increase in cytoplasmic calcium concentration is detected during apoptosis as calcium is released from intracellular stores (Rasola and Bernardi 2007, Kroemer *et al.* 2007, Contreras *et al.* 2010). Additionally, cytoplasmic calcium concentration controls mitochondrial membrane potential. We used Fluo-4 dye to explore the concentration of cytoplasmic calcium during the early steps of CPV infection. Thapsigargin was used as a positive control to induce the release of calcium from intracellular stores to the cytoplasm. Experiment was performed during the entry phase of infection from 30 min to 6 h p.i.. Only minor increase in cytoplasmic calcium concentration was observed at 30 min to 1 h p.i. (II, Fig. 5) implying that major calcium stores ER and mitochondria were intact.

5.6.3 Activation of cell survival signalling through ERK1/2

The observed decrease in mitochondrial membrane potential and increase in the proportion of damaged mitochondria during early steps of CPV infection led us to examine whether CPV infection initiated survival signalling at beginning of the infection. We used immunoblotting analysis of ERK1/2 and phosphorylated ERK1/2 (p-ERK1/2) to examine if this MAPK signalling cascade was activated. Densitometry was utilized to measure the intensity of bands and ratio of p-ERK1/2 to ERK1/2 was calculated. Results indicated that ERK1/2 was activated

at 15 min – 2 h p.i. as ERK ratio increased (II, Fig. 6B). At 2 – 6 h p.i. the amount of activated ERK1/2 declined to the level of mock infected cells.

The importance of ERK1/2 activation in a successful CPV infection was studied by inhibiting the activation of ERK1/2 with a MEK inhibitor U0126. Infection percentage was determined in the presence of U0126 and results demonstrated that infection percentage decreased by 20% when activation of ERK1/2 was inhibited (II, 6C). Together, these results demonstrated activation of ERK1/2 survival signalling in CPV infected cells and the consequence of this activation to successful CPV infection.

Further, the influence of ERK1/2 activation to the mitochondrial membrane potential was tested by inhibiting the activation of ERK1/2 with U0126 during an infection, and analyzing the depolarization of mitochondria with JC-1. By preventing the activation of ERK1/2 the mitochondrial depolarization at 2 h p.i. was partly inhibited (II, 6D). These results indicated that additional signalling routes are participating in rescuing cells from stress situation evoked by the entering virus.

5.7 Induction of long extensions in CPV infected cells

DIC microscopy of CPV infected NLFK and A72 cells revealed long protrusions (I, Fig. 1). These extensions were seen at 48 h p.i. and at 72 h p.i. they were more profound. As extensions were seen in both cell line and extensions fused with neighbouring cells they were thought to have some role in CPV infection. Number and length of protrusions were examined at 24 and 72 h p.i. with DIC microscopy (III, Fig. 1). Analysis of extensions revealed that the number and length of them were greater in CPV infected cells at 72 h p.i. than in mock infected cells (III, Fig. 2 and 3). Some changes were observed already at 24 h p.i. indicating that morphological change started early in infection. Also immunofluorescence microscopy was used to study the distribution of intermediate filaments in these extensions. Extensions were found to contain tubulin (III, Fig 1F, J), but not actin or vimentin (III, data not shown). Interestingly, tubulin was found to concentrate to extensions (III, Fig. 1F). The long protrusions could contribute to the spread of virus.

6 DISCUSSION

CPV is one of the most studied parvoviruses and important aspects of its life cycle have been studied previously, but the mechanism of its cytopathic effect has not been examined in full. In this thesis the interaction of CPV with host cells and the cytopathic effects were determined giving emphasis on parvovirus–mitochondria interactions. Our results demonstrated interesting new data of the cytopathy of the parvoviruses in common. Results enlighten the complicated interactions of the virus and host cell during infection and the multiple outcomes of this. The results concerning the mechanism of cell death are illustrated in the Figure 6 and are discussed in the below paragraphs.

Parvoviruses have cell line dependent effects (Ohshima *et al.* 1998, Sol *et al.* 1999, Di Piazza *et al.* 2007, Chen *et al.* 2010, Poole *et al.* 2011). Our results demonstrated similar effects in both cell lines used, canine A72 cells and feline NLFK cells. The infection proceeded faster in NLFK cells than in A72 cells as detected with beginning of viral protein expression by NS1 protein (I, Fig. 1 and 5A). The observed NS1 expression is dependent on the sensitivity of the method and earlier production of NS1 can not be ruled out. The faster proceeding infection in NLFK cells has made it the main cell line utilized in CPV studies (Vihinen-Ranta *et al.* 1998, Parker and Parrish 2000, Suikkanen *et al.* 2002, Suikkanen *et al.* 2003a, Suikkanen *et al.* 2003b). Accordingly, NLFK cell line was the main cell line in our studies. In addition, part of the experiments was also conducted in A72 cells as it presents the more natural host for the CPV.

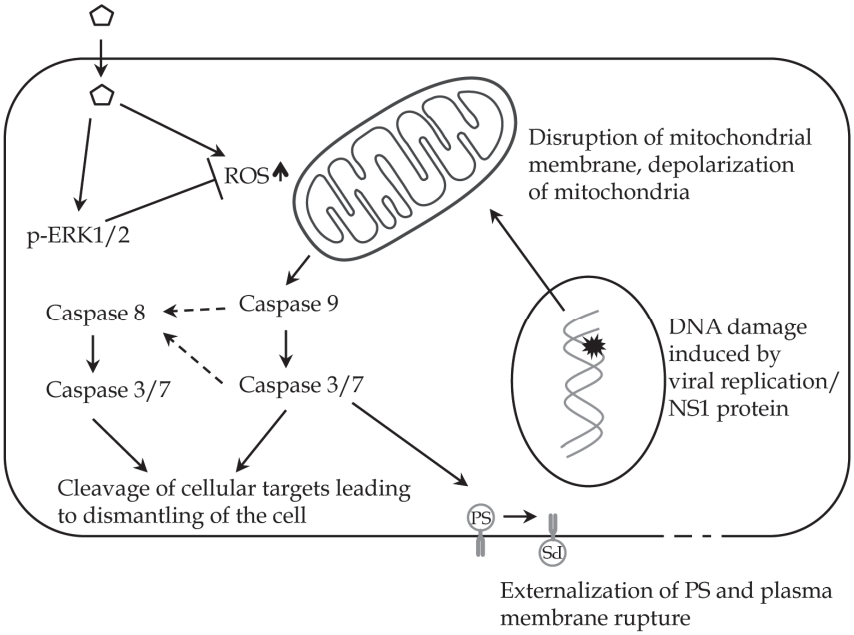

FIGURE 6 The proposed pathway of CPV induced cell death based on the results of this thesis. The entering virus induced oxidative stress that is normalized by activation of cell survival signalling and main inducer for CPV apoptosis is the DNA damage created by viral replication and action of NS1. This signal is transmitted to mitochondria possibly through Bcl-2 family members leading to disruption of mitochondrial membrane potential and release of proapoptotic proteins. Caspase 9 is activated that further activates caspase 3/7. Actions of caspases 9 and 3/7 lead to activation of caspase 8 that additionally activates caspase 3/7. Activated caspase 3/7 cleaves multiple targets leading to externalization of phosphatidylserine, plasma membrane rupture and the death of infected cell. p-ERK1/2, phosphorylated extracellular regulated kinase; PS, phosphatidyl serine; ROS, reactive oxygen species.

6.1 Influence of CPV infection to the cell cycle

Parvoviruses are dependent on the replicative machinery of their host cells. In connection to this parvovirus infection frequently causes cell cycle arrest at S or G_2 even though block at G_1 has also been observed (Oleksiewicz and Alexandersen 1997, Morita *et al.* 2001, Morita *et al.* 2003). We found CPV infection to arrest the cell cycle at the S phase of the cell cycle (I, Fig. 2) as has been reported for ADV and B19V (Oleksiewicz and Alexandersen 1997, Kivovich *et al.* 2012, Luo *et al.* 2013). Additionally, cells with larger than 4N DNA content were observed in our studies (I, Fig. 2). Replication of cellular DNA during S phase doubles the amount of DNA in cell and this 4N DNA content is seen at G_2 (Vermeulen *et al.* 2003). Amount of cells with 4N DNA content increased in B19V infection suggesting arrest at G_2 arrest (Wan *et al.* 2010), but another study revealed that DNA replication is active in these 4N DNA cells and demonstrated

them to be at S phase (Luo *et al.* 2013). Similar pattern has been reported for ADV (Oleksiewicz and Alexandersen 1997). We did not study the proteins controlling the progression of cell cycle or activity of replication but used the amount of DNA to conclude the phase of cell cycle. The amount of cells in S phase increased already from 12 h p.i. and it increased further to 24 h p.i. providing evidence for S phase arrest (I, Fig. 2). If one takes together the amount of cells with a 4N DNA content (G_2 phase) and cells with more than 4N DNA it decreases at 12–16 h p.i. when compared to mock infected cells accounting for S phase arrest. However, at 24 h p.i. the amount of these cells is almost doubled when compared to mock infected cells (I, Fig. 2). By this time new viruses are being made and the extra DNA accounts for viral DNA. The 4N DNA content suggest cells to be at G_2 phase, although we do not know if infected cells leave S phase. The factor inducing the cell cycle arrest during MVM, ADV and B19V infections has been reported to be the expression of NS1 protein (Op De Beeck and Caillet-Fauquet 1997, Oleksiewicz and Alexandersen 1997, Op De Beeck *et al.* 2001, Morita *et al.* 2003, Nakashima *et al.* 2004, Wan *et al.* 2010). In our studies the cell cycle disturbances started to collect at 12 h p.i. in NLFK cells and 24 h p.i. in A72 cells (I, Fig. 2). By these time points expression of NS1 protein has started (I, Fig. 5A) and we can consider that NS1 has a role in inducing cell cycle disruptions in CPV infected cells. Arrest at S phase of cell cycle can be seen favourable for the virus to maximize the viral replication and production of progeny. As cell cycle block enhances the amount of produced viruses it can bee seen as a factor contributing to the severity of infection (Meunier *et al.* 1985). In addition, block frequently leads to cell death (Op De Beeck and Caillet-Fauquet 1997, Morita *et al.* 2001, He *et al.* 2010, Kannan *et al.* 2011). Taken together, our results demonstrate that expression of NS1 in CPV infected cells induces S phase arrest supporting viral replication.

6.2 CPV infection induces an intrinsic cell death pathway

We utilized morphological and biochemical studies to differentiate the cell death type of CPV infected cells. DIC microscopy revealed rounding of the cells and later detachment of cells (I, Fig. 1). Caspase activation is one of the hallmarks of apoptosis (Kumar 2007, Kroemer *et al.* 2009) and our experiments revealed activation of caspases both in NLFK and A72 cells (I, Fig. 4). Caspase 9 was activated early in infection and the activity persisted to later phases of infection. Additionally, caspase 8 was activated during CPV infection but to a lesser extent. Activity of effector caspase 3/7 was detected accounting for cell death through apoptosis. Similar activation of caspases 3/7 and 9 is observed during expression of H–1PV NS1 in human cells and B19V infection or expression of B19V NS1 protein in erythroid progenitor cells and hepatocytes (Moffatt *et al.* 1998, Poole *et al.* 2004, Hristov *et al.* 2010). However, activation of caspase 8 is not detected in B19V infected hepatocytes but this caspase is activated during B19V infection in erythroid progenitor cells (Sol *et al.* 1999, Poole *et al.* 2004). Indeed, the apoptosis

in B19V infected erythroid progenitors proceeds via the extrinsic TNFα pathway. However, activation of caspase 9 was not studied in these cells (Sol *et al.* 1999). In addition, extrinsic pathway is suggested for B19V infection in fully permissive erythroid progenitor cells where activation of caspase 10 was most prominent with minor activation of caspases 8 and 9 (Chen *et al.* 2010). In our studies the major activity of caspase 9 compared to caspase 8 indicates activation of intrinsic apoptotic pathway in CPV infected cells.

Another characteristic event in apoptosis is condensation of chromatin alongside fragmentation of DNA and nucleus. All these events were observed in CPV infected NLFK cells (I, Fig. 5). Phosphorylation of histone H2A.X is an early and accurate marker of DNA double strand brakes (Rogakou *et al.* 2000, Talasz *et al.* 2002, Lu *et al.* 2006). We were the first to demonstrate phosphorylation of H2A.X indicating DNA double strand brakes in parvovirus-infected cells (I). Phosphorylation of H2A.X was detected from 12 h p.i. onwards and it occurred concomitantly with NS1 expression suggesting role for NS1 in inducing host DNA damage (I, Fig. 5A). Later it has been confirmed that indeed expression of CPV NS1 induces DNA damage and apoptosis (Saxena *et al.* 2013). In addition, it has been demonstrated that apoptosis after expression of B19V NS1 causes formation of apoptotic bodies. These apoptotic bodies contain viral antigens alongside self antigens, including DNA, and they could contribute to the breakdown of immune tolerance leading to the autoimmune diseases connected to a B19V infection. Thus, the host DNA damage induced by parvoviral NS1 protein is an important factor in pathology associated with parvovirus infections.

Mitochondrial membrane depolarization is characteristic of disruptions in mitochondrial membrane during apoptosis (Zamzami *et al.* 1995a, Green and Reed 1998, Dussmann *et al.* 2003). Mitochondria began to depolarize in CPV infected cells after 14 h of infection (II, Fig. 3). Involvement of caspase 9 and mitochondrial membrane depolarization argue for intrinsic apoptotic pathway. Together, our results indicate that expression of NS1 induces DNA damage that activates apoptosis through intrinsic pathway (Fig. 6). Similar apoptotic pathway involving DNA damage and activation of caspases 9 and 3 has been reported for B19V in hepatocytes and H–1PV in human cells (Poole *et al.* 2004, Poole *et al.* 2006, Hristov *et al.* 2010, Poole *et al.* 2011).

Caspase 9 is the initiator caspase of intrinsic apoptotic pathway. It is activated by multimerization with apaf-1 in the presence of cytochrome c, which is released from mitochondria after membrane permeabilization. Bcl-2 family proteins regulate this release that is accompanied with depolarization of mitochondrial membrane potential (Chang and Yang 2000, Kumar 2007). In CPV infected NLFK cells caspase 9 was activated already at 6 h p.i. (I, Fig. 4A) and depolarization of mitochondrial membrane potential was detected only after 14 h p.i. (II, Fig. 3). However, it has been reported that cytochrome c release can precede mitochondrial damage (Bossy-Wetzel *et al.* 1998). Release of cytochrome c without depolarization of mitochondrial membrane could explain the activation of caspase 9 in CPV infected cells. ROS is one factor inducing release of cytochrome c from mitochondria (Zamzami *et al.* 1995b). We detected increase in

intracellular ROS at 2-6 h p.i. (II, Fig. 4) that was accompanied with depolarization of some mitochondria at 2 h p.i. (II, Fig. 3). Additionally electronmicroscopy studies of mitochondrial ultrastructure revealed increase in the proportion of damaged mitochondria at 2 h p.i. but the proportion had decreased at 6 h p.i. being nevertheless about 10% more than in mock infected cells (II, Fig. 2). These results indicate damage to a small population of mitochondria at the very early infection that could explain the activation of caspase 9 before the final depolarization of mitochondria and DNA damage. In earlier studies fragmentation of DNA was needed for depolarization of mitochondria (Bossy-Wetzel *et al.* 1998). Similarly in our studies the depolarization of mitochondria followed the DNA damage (detected at 12 h p.i.) (II, Fig. 3, I, Fig. 5A) suggesting that a signalling cascade is activated by DNA damage leading to activation of the main intrinsic apoptotic pathway and enhancing activation of caspase 9. The formation of ROS during early phases of infection and subsequent normalization of ROS level in our studies differs from accumulation of ROS in human cells expressing H–1PV NS1. The H–1PV NS1 induced accumulation of ROS was evidently involved in the induction of DNA damage and apoptosis (Hristov *et al.* 2010) that was not detected in our studies (I, Fig. 5, II, Fig. 4).

In addition to the activation of the caspase 9, the initator caspase 8 was activated during CPV infection (I, Fig. 4). Caspase 8 is activated during extrinsic apoptotic pathway as a response to the activation of the death receptors (Chang and Yang 2000, Kumar 2007). However, caspase 8 has been reported to be activated by active caspase 3 in apoptosis independent of death receptors and additionally caspase 9 may activate caspase 8 (Slee *et al.* 1999, Tang *et al.* 2000). In CPV infected cells the activation of caspase 8 occurred concomitantly (NLFK) or after (A72) activation of caspase 3 and after activation of caspase 9 (I, Fig. 4) suggesting a role for caspases 3 and 9 in the activation of caspase 8 (Fig. 6).

Activation of caspase 9 before major disruption of mitochondria, as detected with mitochondrial membrane potential, led us to investigate whether CPV directly interacts with mitochondria. Such an interaction has been reported for other viruses like HCV, HIV-1, respiratory syncytial virus and rotavirus but not with parvoviruses (Korenaga *et al.* 2005, Chu *et al.* 2011, Huang *et al.* 2012, Boyapalle *et al.* 2012, Bhowmick *et al.* 2012). Damage to the mitochondrial membrane was not observed when electron microscopy was utilized to detect damage to the nuclear envelope after microinjection of parvovirus MVM. Neither did incubation of isolated mitochondria with MVM damage mitochondria (Cohen and Pante 2005). However, these studies with MVM were conducted in the absence of viral replication and expression of viral protein. We studied the interaction of CPV with the mitochondria in infected cells throughout the viral lifecycle. Colocalization analysis based on confocal microscopy images demonstrated that CPV colocalized with mitochondria (II, Fig. 1). Conversely, a closer examination with electron microscopy revealed that CPV was associated with mitochondrial outer membrane but did not penetrate into mitochondria (II, Fig. 2). More damaged mitochondria were observed in all CPV infected samples than in mock infected samples at all examined time points. At 10 h p.i. a more

profound damage was observed. This indicates that even though CPV associates with mitochondria it is not the principal trigger for disruption of mitochondria. Thus, as more significant damage leading to depolarization of mitochondrial membrane potential was detected at 10 h p.i., it can be concluded that activation of viral replication, expression of NS1 and thereafter DNA damage is the main trigger for apoptosis in CPV infected cells.

In vivo phagocytic cells engulf and degrade the remains of late apoptotic cells (Kroemer *et al.* 2009). To mark the dying cell for phagocytosis phosphatidylserine is translocated to the outer leaflet of plasma membrane (Martin *et al.* 1995). Labelling of phosphatidylserine with Annexin V together with PI labeling demonstrated expected Annexin V labeling accounting for apoptosis but additionally cells were labelled with PI (I, Fig. 3). PI is incapable to cross intact plasma membrane (Darzynkiewicz *et al.* 1994) and the results of our experiments revealed that the plasma membrane is affected during CPV infection. Plasma membrane disruption was evident in small proportion of NLFK cells at 12 h p.i. and even 50 % of NLFK cells were labelled with PI after 24 h of infection (I, Fig. 3). Similar observations only with later time points were obtained with A72 cells. Interestingly these cells appeared only slightly rounded with DIC microscopy accounting for a minor alteration of plasma membrane. Rounded cells detached from the culture dish without formation of apoptotic bodies frequently seen in late apoptotic cells (Zhang *et al.* 1999). Indeed, it has been reported that apoptotic bodies are not formed in all apoptotic cells (Taylor *et al.* 2008). In contrast to our results, apoptotic bodies has been detected in HepG2 cells expressing B19V NS1 protein (Thammasri *et al.* 2013).

If cells capable to engulf apoptotic cells are absent, apoptosis converts to necrosis. Necrosis after the onset of apoptosis has been considered as secondary necrosis and has been presumed to happen only if scavenger cells are absent (Majno and Joris 1995, Silva *et al.* 2008). However, it has been reported to occur not only *in vitro* but additionally *in vivo* in multicellular organisms suggesting that secondary necrosis is utilized in termination of apoptosis if scavenger cells fail to clear the dying cell and may account for pathological outcome of apoptosis (Majno and Joris 1995, Silva *et al.* 2008, Krysko *et al.* 2008, Silva 2010). The observed PI labeling in CPV infected cells suggested that CPV infection triggered initially apoptotic cell death of infected cells but at later time points apoptosis turned to secondary necrosis as the plasma membrane was affected. Whether this is due to absence of cells capable of phagocytosis or the main termination pathway in CPV induced apoptosis remains to be studied. With respect to parvoviruses, a BPV infection induces necrosis but, contrast to our studies, apoptotic characteristics were not detected (Abdel-Latif *et al.* 2006). Features of apoptosis before necrotic cell death has been observed during a H–1PV infection (Ran *et al.* 1999). However, in studies with H–1PV the change from apoptosis to necrosis occurred earlier and e.g. caspases were not activated. The switch to necrosis in H-1PV infected cells occurs presumably because of metabolic changes and depletion of energy (Ran *et al.* 1999). Since mitochondria are affected during a CPV infection, the production of energy is disturbed. This could account for induction of secondary necrosis in CPV infection since apoptosis is energy

consuming (Leist *et al.* 1997). The pathological consequence of cell death is different after apoptotic and necrotic cell death. Necrosis involves release of cytoplasmic contents to the extracellular space and induces inflammation while phagocytic cells engulf the remains of apoptotic cell and inflammation is not triggered (Galluzzi *et al.* 2007). Necrosis has been observed in tissue sections of CPV infected dogs intestine but later examination of apoptotic markers from histological samples proved at least some of the CPV infected cells to be apoptotic (Meunier *et al.* 1985, Bauder *et al.* 2000). Thus, it is possible that both types of cell death are occurring during a CPV infection and account for the injury in the epithelia of small intestine and development of enteritis. In addition to tissue damage in intestine, CPV infection affects the lymphoid tissue and cardiomyocytes. Apoptosis of lymphocytes accounts for lymphopenia and extensive cell death of cardiomyocytes that produces cell debris induces fetal myocarditis in puppies.

6.3 Activation of ERK1/2 signalling in CPV infected cells

Experiments with confocal and electron microscopy revealed association of CPV with mitochondria from the early time points on (II, Fig. 1 and 2). Furthermore, electron microscopy studies demonstrated damage to mitochondria during the entry phase of CPV life cycle (II, Fig.2B). Damage to mitochondria at 2 h p.i. was accompanied with depolarization of mitochondrial membrane potential (II, Fig. 3) and increase in ROS level (II, Fig. 4). These results indicate that invading virus affects cellular homeostasis and induces cellular responses like oxidative stress (Fig. 6). Interestingly stress situation is compensated quite shortly as mitochondrial membrane potential was normalized by 4 h p.i. (II, Fig. 3) and the proportion of damaged mitochondria had decreased by 6 h p.i. (II, Fig. 2B). ROS level continued to increase to 6 h p.i. and was then quickly decreased to the level of mock infected cells by 8 h p.i. (II, Fig. 4). Stabilization of the mitochondrial stress induced by entering virus accounts for activated survival signalling. Indeed, activation of ERK1/2 signalling pathway was detected immediately after entry of CPV (II, Fig. 6B). ERK1/2 signal cascade mediates extracellular signals to nucleus and controls expression of multiple proteins involved in cell survival, proliferation and differentiation (Roskoski 2012). Overexpression of ERK1/2 has been detected in many tumour cells to promote survival (Wu *et al.* 2004, Balmanno and Cook 2009). However, prolonged activation of ERK signalling together with atypical cellular location promotes apoptosis (Murphy and Blenis 2006, Lu *et al.* 2010, Cagnol and Chambard 2010). In CPV infected cells the activity of ERK1/2 lasted for 1 h (II, Fig. 6B). Additionally, the observed normalization of the mitochondrial membrane potential (II, Fig. 3) suggests that the role of ERK1/2 signalling during CPV infection is to prevent cell death and activated ERK1/2 signals for survival of the cell (Fig. 6). Similar rapid and short activation of ERK1/2 as detected in CPV infection has been earlier reported to rescue cells from apoptosis triggered by oxidative stress (Wang *et al.* 1998, Chen

et al. 2005, Liang *et al.* 2013). Normalization of mitochondrial homeostasis ensures also the production of energy and metabolites for assembly of new viruses. Hereby it affects the cytotoxic outcome of the infection and the pathological consequence.

Proportion of infected cells decreased by 20% when activation of ERK was inhibited with U0126 (II, Fig. 6C). This result provides evidence that activation of signalling cascade mediated by ERK1/2 is needed for optimal proliferation of virus. However, additional signalling pathways are participating in rescuing cells from stress situation provoked at first stages of infection, as CPV was able to infect cells when the inhibitor of ERK1/2 activation was present (II, Fig. 6C). When mitochondrial membrane depolarization was determined from cells incubated with U0126, a decrease in depolarization of mitochondria was detected (II, Fig. 6D). The result suggests involvement of additional survival signalling pathways in CPV infected cells. These may include PI3K/AKT or pathways inhibiting apoptosis e.g. by regulating Bcl-2 family proteins (Brumatti *et al.* 2010). According to the results obtained from the experiments of ERK1/2 activation during CPV infection we can conclude that entering CPV interacts with cellular signalling cascades to activate cellular survival signalling, including ERK1/2, to prevent cell death before replication of the virus (Fig. 6). Alterations in cellular signalling by invading virus may be broader as inhibition of ERK1/2 activation reduced the depolarization of mitochondrial membranes. This is surprising as overexpression of ERK1/2 inhibits mitochondrial permeability transition (Scheid *et al.* 1999, Chen *et al.* 2008, Pucci *et al.* 2009). Our study was the first to demonstrate the early interference of parvoviruses with the cellular homeostasis leading to the activation of cell survival signalling. CPV induced changes in cellular signalling obviously affect the functions and metabolism of the cell, but more importantly it allows replication of CPV and the influences of this including cell cycle arrest and cell death accounting for tissue damage in an infected dog.

6.4 Induction of long protrusions at the late infection

Parvoviruses have been suggested to be released by lysis of the host cell (Richards *et al.* 1977). This conclusion is justified by the cytotoxicity seen in a parvovirus infection (Moffatt *et al.* 1998, Corbau *et al.* 2000, Nuesch and Rommelaere 2006, Poole *et al.* 2006, Nuesch and Rommelaere 2007, Kivovich *et al.* 2010, Poole *et al.* 2011, Kivovich *et al.* 2012). However, non-lytic release alongside lytic infections has been reported for other non-enveloped viruses e.g. coxsackievirus B3, poliovirus and echovirus (Zhang and Racaniello 1997, Jackson *et al.* 2005, Paloheimo *et al.* 2011). Examination of CPV infected cells at 24 h p.i. and later revealed long protrusions that fused with neighboring cells (III, Fig. 1). The number and length of protrusions started to increase at 24 h p.i. but was more profound at 72 h p.i. (III, Fig. 2 and 3). Formation of protrusions was observed both in NLFK and A72 cells. Similar protrusions have been reported to

mediate direct cell-to-cell spread of viruses including coxsackievirus B3, alphaherperviruses, Marburgvirus and retroviruses (Favoreel *et al.* 2005, Kolesnikova *et al.* 2007, Sherer *et al.* 2007, Paloheimo *et al.* 2011, Nikolic *et al.* 2011). Indeed, immunofluorescence microscopy revealed CPV capsids in protrusions (III, Fig. 1J). Additionally, immunofluorescence experiments indicated that protrusions contained tubulin but not actin or vimentin (III, data not shown). CPV induced protrusions resembled significantly those induced by pseudorabiesvirus with the exception that protrusions in pseudorabies infected cells contained both actin and tubulin (Favoreel *et al.* 2005). However, the distribution of tubulin in both CPV and pseudorabiesvirus induced protrusions were similar locating tubulin at the rim of the cell and in protrusions.

Studies with MVM have demonstrated that actin cytoskeleton is affected during infection while the microtubules are protected and reorganizing of actin filaments by gelsolin is involved in viral egress (Nuesch *et al.* 2005, Bär *et al.* 2008). Egress of MVM has been reported to be a controlled event involving vesicular transport through ER, Golgi and LAMP-2 positive vesicles to plasma membrane and to be essential for maturation of capsids and affect the lysis of the cell (Bär *et al.* 2008, Bär *et al.* 2013). Tubulin is involved in the entry of CPV transporting capsids towards nucleus (Suikkanen *et al.* 2003b). Results obtained in this thesis suggest that transport along tubulin is involved additionally in viral egress. Although results from MVM studies point to the role of actin in parvoviral egress they additionally discuss involvement of tubulin network in transport of MVM containing vesicles towards plasma membrane (Bär *et al.* 2008). By combining the results of Bär *et al.* (2008, 2013) and ours we can speculate that the egress of CPV (probably in vesicles) proceeds along microtubules in the long protrusions. This provides direct spread from an infected cell to a non-infected one and prevents the host immune response toward the infection. Interestingly studies with B19V have indicated that infection enhances the motility of infected synovial fibroblasts. Infected cells were more invasive and they were able to migrate through cartilage (Ray *et al.* 2001). This change in cellular behavior provides a mechanism how B19V infection could induce arthritis (Takahashi *et al.* 1998, Ray *et al.* 2001). Long protrusions contribute to the migration of cells (Bravo-Cordero *et al.* 2012) and viral induction of these is connected to the cell transformation (Taylor *et al.* 2011). The long protrusions observed in our studies (III) and the invasiveness of B19V infected cells (Ray *et al.* 2001) suggest that cytoskeletal rearrangements could be a more common feature during parvovirus infection contributing to the pathology seen in parvovirus infections.

7 CONCLUSIONS

The main conclusions of this thesis are:

1. The cytopathic effects of CPV infection include arrest at the S phase of cell cycle. Furthermore, CPV infection induces apoptotic cell death that proceeds through the intrinsic pathway. Apoptosis is triggered after the beginning of viral replication by NS1-induced DNA damage, and apoptotic program includes activation of caspases and mitochondrial damage.
2. The entering virus induces mitochondrial stress in host cells. However, the cells later overcome this mitochondrial stress and the virus is able to continue its lifecycle. Survival signalling involving the ERK1/2 pathway participates in the normalization of cellular homeostasis.
3. CPV infection induces formation of long protrusions in infected cells. Protrusions may have a role in egress of viral progeny.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Viruksen ja solun väliset vuorovaikutukset parvovirusinfektion patologisena mekanismina

Virukset ovat pieniä partikkeleita, jotka ovat täysin riippuvaisia soluista. Ne hyödyntävät infektoimansa solun koneistoja omassa lisääntymisessään. Viruksen lisääntyminen voi johtaa häiriöihin isäntäsolun toiminnoissa ja jopa isäntäsolun kuolemaan aiheuttaen sairauden infektoidussa eliössä. Viruspatologia tutkii näitä sairauden kehittymiseen johtavia mekanismeja. Nykyään viruksia pystytään myös hyödyntämään lääketieteessä. Yksi esimerkki tästä on geeniterapia, jossa viruksen avulla voidaan kuljettaa soluihin tautia hoitavaa proteiinia koodaava geeni. Onkolyyttisessa virusterapiassa puolestaan hyödynnetään virusten kykyä tappaa infektoimansa solu. Virusten ja niiden isäntäsolujen monimutkaiset vuorovaikutussuhteet on tunnettava, jotta virusten aiheuttamia tauteja voitaisiin hoitaa tehokkaasti, sekä hyödyntää viruksia lääketieteen työkaluina.

Viruksen tunkeutuminen soluun ja lisääntyminen häiritsevät monia solun toimintoja. Virus käyttää solun tuottamaa energiaa ja makromolekyylejä, jolloin niitä ei riitä tarpeeksi solun omiin toimintoihin. Virus voi myös omilla proteiineillaan tai toimintansa kautta aktivoida tai estää solun signaalinvälitysreittejä. Hyvin usein viruksen lisääntyminen saa aikaan apoptoosin infektoidussa solussa. Apoptoosi on solukuolema, joka etenee kontrolloidusti tiettyjen vaiheiden kautta ja päättyy solun hajoamiseen pieniin solukalvon ympäröimiin rakkuloihin, jotka läheiset solut tuhoavat. Tällä tavalla solun sisältö ei vapaudu ympäröivään kudokseen ja vältytään tulehdusreaktiolta. Virusinfektiossa solukuolema kuitenkin aiheuttaa kudostuhoa, joka on usein suoraan kytköksissä viruksen aiheuttamaan sairauteen. Näin on esimerkiksi tässä tutkimuksessa käytetyn koiran parvoviruksen (canine parvovirus, CPV) kohdalla. CPV-infektio johtaa infektoidussa koirassa mm. suolen epiteelisolujen tuhoutumiseen, mikä ilmenee suolistotulehduksena.

Virusten koko vaihtelee muutamasta kymmenestä nanometristä muutamaan sataan nanometriin. Tässä väitöskirjatutkimuksessa käytetty CPV kuuluu parvoviruksiin, jotka lukeutuvat yksinkertaisimpiin viruksiin. Parvovirus koostuu proteiinikuoresta, jonka sisään on suljettu sen yksisäikeisestä DNA:sta koostuva perintöaines. CPV-partikkeli on halkaisijaltaan vain n. 30 nm ja sen genomissa on kaksi geeniä. Parvovirukset infektoivat monia eläimiä. Niistä kaksi, parvovirus B19 ja ihmisen bocavirus, aiheuttavat sairauksia ihmisille.

Tässä väitöskirjatyössä tutkittiin CPV:n ja isäntäsolun solutuhoa aiheuttavia vuorovaikutusmekanismeja. Saadut tulokset varmistivat CPV-infektion aiheuttavan apoptoosia infektoiduissa soluissa. Väitöskirjatyössä saadut tulokset osoittivat mitokondrion kalvopotentiaalin heikkenemisen ja reaktiivisten happiradikaalien muodostumisen infektion alkuvaiheiden aikana. Nämä muutokset liittyvät mitokondrionaaliseen stressireaktioon, joka voi johtaa solukuolemaan. Stressitilanne kuitenkin normalisoitui nopeasti CPV-infektoiduissa so-

luissa. Tilanteen normalisoituminen näytti liittyvän tiettyjen signalointireittien aktivoitumiseen. Yksi näistä on ERK1/2-signalointireitti, jonka aktivoituminen havaittiin koiran parvoviruksen infektoimissa soluissa. Viruksen soluun tunkeutumisen aiheuttaman stressin normalisointi estää solua kuolemasta infektion aikaisessa vaiheessa ja mahdollistaa viruksen lisääntymisen.

Infektion edettyä viruksen perintöaineksen monistumiseen ja proteiinisynteesin käynnistymiseen, jonka ajoittamisessa hyödynnettiin viruksen NS1-proteiinin ilmestymistä soluihin, infektion havaittiin pysäyttävän solusyklin synteesivaiheeseen. Tässä solusyklin vaiheessa solun replikaatiokoneisto on aktiivinen, mikä edelleen tukee viruksen lisääntymistä. NS1-proteiinin ilmaantumisen jälkeen havaittiin myös apoptoosille tyypillisiä tapahtumia, kuten kaspaasien aktivoitumista. Kaspaasit ovat entsyymejä, jotka välittävät solun apoptoottista kuolemaa hajottamalla solulle elintärkeitä proteiineja ja toimintoja. Näiden proteiinien aktivoituminen havaittiin infektion aikana. Lisäksi havaittiin vaurioita mitokondrion kalvolla, solukalvolla sekä DNA:ssa. Vauriot mitokondrioissa ja DNA:ssa viittaavat mitokondriovälitteisen apoptoosireitin aktivoitumiseen. Fluoresenssimikroskooppitutkimukset osoittivat, että osa CPV-partikkeleista sijaitsee soluissa lähellä mitokondrioita. Tarkempi tarkastelu elektronimikroskopialla osoitti CPV:n jopa olevan vuorovaikutuksessa mitokondrion uloimman kalvon kanssa infektion alkuvaihesta lähtien. Tämä vuorovaikutus ei kuitenkaan ollut ratkaiseva tekijä mitokondrioiden vaurioittamisessa ja solukuoleman laukaisemisessa, sillä varsinaiset apoptoottiset muutokset soluissa havaittiin NS1-proteiinin ilmaantumisen jälkeen. Tämä viittaa NS1-proteiinin osuuteen solukuoleman aiheuttamisessa. Tässä väitöskirjatyössä saatujen tulosten perusteella voidaan päätellä CPV-infektion aiheuttavan apoptoottisen solukuoleman, joka mahdollisesti saa alkunsa NS1-proteiinin aiheuttamasta DNA-vauriosta. Signaali vaurioituneesta DNA:sta välitetään mitokondrioon, joka aktivoi apoptoottisen koneiston ja saa aikaan solun tuhoamisen.

CPV-infektion havaittiin myös vaikuttavan merkittävästi infektoitujen solujen rakenteeseen pitkissä infektioissa. Solut muodostivat pitkiä ulokkeita, joiden sisällä havaittiin viruskapsideja. Tutkimukset osoittivat myös, että ulokkeissa on solun tukirangan tubuliinisäikeitä. Samanlaisia ulokkeita on havaittu myös muiden virusten infektioiden yhteydessä ja ulokkeiden on todettu osallistuvan virusten leviämiseen. CPV-infektiossa ulokkeilla saattaa olla samantyyppinen merkitys viruksen leviämisessä.

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ORIGINAL PAPERS

I

MECHANISMS OF CELL DEATH IN CANINE PARVOVIRUS-INFECTED CELLS PROVIDE INTUITIVE INSIGHTS TO DEVELOPING NANOTOOLS FOR MEDICINE

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II

ROLE OF MITOCHONDRIA IN PARVOVIRUS PATHOLOGY

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Role of Mitochondria in Parvovirus Pathology

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Abstract

Proper functioning of the mitochondria is crucial for the survival of the cell. Viruses are able to interfere with mitochondrial functions as they infect the host cell. Parvoviruses are known to induce apoptosis in infected cells, but the role of the mitochondria in parvovirus induced cytopathy is only partially known. Here we demonstrate with confocal and electron microscopy that canine parvovirus (CPV) associated with the mitochondrial outer membrane from the onset of infection. During viral entry a transient depolarization of the mitochondrial transmembrane potential and increase in ROS level was detected. Subsequently, mitochondrial homeostasis was normalized shortly, as detected by repolarization of the mitochondrial membrane and decrease of ROS. Indeed, activation of cell survival signalling through ERK1/2 cascade was observed early in CPV infected cells. At 12 hours post infection, concurrent with the expression of viral non-structural protein 1, damage to the mitochondrial structure and depolarization of its membrane were apparent. Results of this study provide additional insight of parvovirus pathology and also more general information of virus-mitochondria association.

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Introduction

Mitochondria are important organelles for the cell as they produce energy, regulate redox balance and maintain Ca^{2+} homeostasis. In cell signalling the mitochondria regulate cell responses to different cellular situations determining the fate of cell from survival to death [1–3]. In viral infections, mitochondria have a role in innate immunity by activating interferon production [4]. Mitochondrial dysfunction is associated with numerous diseases such as neurodegenerative diseases, diabetes and cancer [5–11]. Among the factors leading to mitochondrial dysfunction are depolarization of the mitochondrial transmembrane potential ($\Delta\Psi$m), changes in expression of mitochondrial proteins and lipids, mutations in mtDNA, oxidative stress, and alterations in mitochondrial number [5–11].

Many viral proteins target the mitochondria and interfere with its functions contributing to pathology of viral diseases [12,13]. For example, association of hepatitis C virus (HCV) proteins with the mitochondria play an important role in pathogenesis of HCV induced chronic liver diseases and liver cancer. HCV proteins enter the mitochondria causing an increase in mitochondrial Ca^{2+} uptake, reactive oxygen species (ROS) production and mitochondrial permeability transition. As a result, intrinsic cell death and changes in the liver microenvironment lead to cell transformation [14,15]. One major factor in HIV pathogenesis is viral protein R (Vpr). Vpr is integrated in the mitochondrial outer membrane and it also reduces the expression of mitofusin 2, which leads to mitochondrial fragmentation and depolarization of $\Delta\Psi$m inducing death of infected $CD4^+$ T lymphocytes [16]. On the other hand, respiratory syncytial virus (RSV) can cause severe infections as viral non-structural protein 1 (NS1) interferes with mitochondrial antiviral signalling protein inhibiting the interferon production [17]. Immune response is therefore delayed early in an RSV infection giving more time for viral replication.

Viruses can modulate mitochondrial functions for their benefit and they can interfere with signalling networks activating growth pathways to increase metabolic activity [18,19]. One example is the activation of phosphatidylinositol-3 kinases/AKT (PI3K/AKT) survival pathway by rotaviral non-structural protein 1 (NSP1) in the beginning of infection [20]. Another rotaviral protein, NSP4, is integrated into the mitochondrial membranes causing apoptosis through depolarization of mitochondria and release of cytochrome c [20]. NSP1 counteracts the NSP4 induced apoptosis early in the infection giving time for viral replication. Another survival signalling pathway is mediated through the extracellular regulated kinases 1 and 2 (ERK1/2). ERK1/2 signal cascade activates cytoplasmic and nuclear substrates that promote cell survival, cell division, differentiation and cell motility [21]. Overexpression of ERK1/2 has been reported to inhibit the intrinsic mitochondria dependent apoptotic pathway [22]. As a results of its functions, activation of ERK1/2 signalling has been reported to be important mediator in pathogenesis of number of viruses including echovirus 1 [23], coxsackievirus B3 [24], entrovirus 71 [25], vaccinia virus [26], human cytomegalovirus [27], influenza virus [28] and HIV-1 [29]. During virus infection the significance of ERK1/2 activation is mainly to prevent apoptosis and ensure production of viral progeny.

Parvoviruses are small non-enveloped viruses with linear ssDNA genome [30]. Pathology of parvoviral infection is often directly connected to the cytotoxic nature of infection. Enteritis, myocarditis, hepatitis and reticulocytopenia are consequences of parvovirus induced cell death [31–35]. The mechanisms of cell death have been reported to be apoptosis, necrosis and death by cytoskeletal rearrangements [34,36–40]. We have used canine parvovirus (CPV) as a model virus in our studies concerning parvovirus pathology and we have earlier reported that a CPV infection initiates apoptosis [41]. CPV induced apoptosis involves activation of caspases (9 and 3) and dissipation of $\Delta\Psi$m. Caspases

are activated early in the infection, but damage to nuclear DNA and depolarization of $\Delta\Psi$m appear after beginning of viral replication [41]. Others have reported that expression of CPV NS1 protein induces apoptosis in a p53 and Bcl-2 independent fashion [42].

Studies up to this point have reported the events of viral induced apoptosis from late stages of infections. Our aim here is to examine the role of the mitochondria in parvovirus pathology at very early stages of infection. The direct association of CPV with the mitochondria and the consequence of this immediate interaction could potentially be the initial triggers for intrinsic cell death. Alternatively, the NS1 expression and its subsequent cytopathic effects on the cell could also be the original trigger. With both possibilities, a comparison of viral-mitochondrial involvement of early events with later events is reported. Our results demonstrate a close mitochondrial association of CPV, and damage to the morphology of the mitochondria as well as induction of oxidative stress. Interestingly a biphasic compromise of $\Delta\Psi$m was observed. More significantly, we report the activation of ERK1/2 signalling in parvovirus infection. Knowledge of exact triggers of intrinsic cell death from viral infections will allow better therapeutic interventions and possible alleviation of pathological consequences.

Materials and Methods

Cells and virus

Norden laboratory feline kidney (NLFK) cells [43] (gift from Colin Parrish, Cornell University, Ithaca, N.Y.), permissive to CPV, were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, CA, USA) supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria) and 1% PenStrep (Invitrogen Life Technologies, CA, USA). Canine parvovirus type 2 (gift from Colin Parrish, Cornell University, Ithaca, N.Y., USA) derived from an infectious clone, as previously described [44], was propagated in NLFK cells in 500 cm^2 cell culture flasks (Nunc, Roskilde, Denmark) for 7 days and then stored at -20°C. Cell debris was removed from 300 ml of virus culture medium by centrifugation and the supernatants were concentrated by ultrafiltration (30 kDa filter, Merck Millipore, Darmstadt, Germany). The virus was pelleted by ultracentrifugation at 173 000× g for 1 h and resuspended in 1 ml of PBS pH 7.4. The suspension was sonicated with low power and extracted with chloroform. Full and empty capsids were separated from the CPV containing aqueous layer by isopycnic centrifugation in 45% cesium chloride gradient. Opalescent bands were collected with a syringe and capsids were pelleted by ultracentrifugation at 245 000× g for 4 h. The pellets were resuspended in 100 µl of PBS. Full CPV capsids were used to infect NLFK cells at m.o.i. 10.

Immunofluorescence microscopy

To study colocalization of CPV with mitochondria, fluorescence confocal microscopy was used. NLFK cells were grown on coverslips and synchronized with 2 mM Thymidine (Sigma-Aldrich, St. Louis, MO, USA) for 18 h. Cells were rinsed with DMEM and inoculated with CPV (m.o.i. 10) in a small volume for 15 min. Then DMEM was added and cells were incubated for 2–24 h. Mock and CPV infected cells were stained with 300 nM MitoTrackerRed (MTR, Invitrogen Life Technologies, CA, USA) for 30 min at 37°C. Cells were fixed with 4% paraformaldehyde, treated with 0.1% Triton in PBS and CPV capsid proteins were labelled with monoclonal anti-CPV antibody (gift from Colin Parrish, [45]) diluted in Triton solution at concentration of 10 µg/ml. Anti-CPV antibody was visualised with Alexa Fluor 488 conjugated anti-mouse antibody (Invitrogen Life Technologies, CA, USA) at a dilution of 1:200. To label mitochondria with polyclonal anti-COX IV antibody (Abcam, Cambridge, UK) mock and CPV infected NLFK cells grown on coverslips were fixed with ice cold methanol and double labelled with monoclonal mouse anti-CPV antibody and with anti-COX IV antibody at a concentration of 7 µg/ml. Alexa Fluor 488 conjugated anti-mouse antibody and Alexa 594 conjugated anti-rabbit antibody (dilution 1:200) were used to detect primary antibodies. The samples were examined with a laser scanning fluorescence microscope (LSM 510, Axiovert 100 M; Zeiss, Jena Germany) by using the excitation and emission settings appropriate for the dye used. Single confocal sections were taken from the middle of the cell.

Colocalization analysis

To quantify the level of colocalization, 10 cells per time point from three independent experiments, 30 cells per time point all together, were randomly selected and imaged using confocal microscope. Levels for the laser power and detector amplification were optimized for each channel in the confocal microscope before starting the quantification. The nucleus was excluded from the image for colocalization analysis. Quantification of colocalization was determined with BioImageXD software [46]. Overlap between channels was expressed as percentage Ch2 voxels colocalizing with Ch1 voxels. The colocalization thresholds were set manually to eliminate background fluorescence and all connected regions with fewer than three pixels were removed to eliminate photon shot noise.

Immunoelectron microscopy

Cells were grown on 8.8 cm^2 plastic culture dishes (Nunc, Roskilde, Denmark) to 80% confluency. The cells were synchronized and infected as stated above. After infection dishes were washed with 0.1 M phosphate buffer, pH 7.4 and the cells were fixed with PLP-fixative (0.01 M sodium periodate, 0.075 M lysine, 2% PFA) for 2 h at RT and left in 2% PFA overnight. After rinsing with sodium phosphate buffer, cells were permeabilized for 8 min at RT with phosphate buffer containing 0.01% saponin and 0.1% BSA. Primary antibody (anti-CPV antibody, used at concentration 10 µg/ml) and gold-conjugated secondary antibody (dilution 1:50; British Biocell International, Cardiff, UK) were diluted in permeabilization buffer and incubated with cells for 1 h at RT. Between and after labelings, cells were washed with permeabilization buffer. Cells were postfixed with 1% glutaraldehyde in 0.1 M phosphate buffer for 10 min at RT, quenched with 50 mM NH_4Cl in phosphate buffer, and washed with both phosphate buffer and water. Cells were treated in the dark with HQ-silver (British Biocell International, Cardiff, UK) for 2 min, followed by washes with water and gold toning solutions [2% sodium acetate 3×5 min, 0.05% gold chloride (Sigma-Aldrich, St. Louis, MO, USA) 10 min on ice, 0.3% sodium thiosulphate 2×10 min on ice]. After washes with water, the cells were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h at 4°C, dehydrated with a descending concentration series of ethanol, and stained with 2% uranyl acetate. Plastic capsules filled with Epon LX-112 (Ladd Research Industries, Williston, VT, USA) were placed upside-down on top of the cells. Epon was polymerized for 24 h at 45°C and 24 h at 60°C. After polymerization, the capsules were warmed up to 100°C and removed carefully. Horizontal sections were cut, picked up on a grid, and viewed with an electron microscope JEM-1400 (Jeol, Tokyo, Japan). Images were taken with iTEM software (Olympus, Münster, Germany). To analyze the impact of CPV infection on mitochondria, all mitochondria from one cell were classified as

intact or damaged and counted. All together mitochondria from 30 cells per time point from three independent experiments were counted.

Flow cytometric analyses of membrane integrity

To analyse if a CPV infection has an effect on $\Delta\Psi$m at early time points p.i., JC-1 (Invitrogen Life Technologies, CA, USA) was used. JC-1 is a fluorescent dye that is used to detect the loss of $\Delta\Psi$m. In healthy cells the dye monomers aggregate and mitochondria can be seen as red, but when mitochondria are depolarized the dye is in a monomeric form and emits green fluorescence. NLFK cells grown in 8.8 cm^2 dishes (Nunc, Roskilde, Denmark) were synchronized infected (or mock infected) with CPV and incubated for 2–18 h. JC-1 was added to trypsinized cells at a final concentration of 15 μM and incubated for 30 min at 37°C. As a control cells were also labelled in the presence of 100 nM K^+-selective ionophore valinomycin (Sigma-Aldrich, St. Louis, MO, USA) that collapsed $\Delta\Psi$m. Stained cells were detected with flow cytometry using a FACSCalibur with a 488 nm laser line and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA) and results were further analysed with FlowJo software (Tree Star, Ashland, OR, USA). A total of 10^4 cells were considered in each assay to create the cytograms. Cells were gated to two populations according to valinomycin treated cells (depolarized mitochondria) and mock infected cells (polarized mitochondria). Amount of cells showing depolarization of $\Delta\Psi$m were normalized in comparison with the mock infected control, which was given the value 1. These experiments were repeated three times.

Detection of ROS

The level of intracellular reactive oxygen species (ROS) was determined with DCFDA cellular ROS detection assay kit (Abcam, Cambridge, MA, USA). Synchronized and infected cells were trypsinized and stained with 20 μM DCFDA for 45 min at 37°C. To induce formation of ROS in positive control, cells were incubated for 2 h with 50 μM tert-butyl hydroperoxide (TBHP) or with 0.5 μM staurosporine (STS) for 16 h. Samples were analysed with flow cytometry using a FACSCalibur with a 488 nm laser line and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ, USA). Results were further analysed with FlowJo software (Tree Star, Ashland, OR, USA). A total of 10^4 cells were considered in each assay to create the cytograms. Cells were gated to two populations: first population corresponded to the production of ROS as compared to TBHP and STS treated cells; and the second population according to mock infected cells. The amount of cells positive for ROS were normalized in comparison with the mock infected control, which was given the value 1. These experiments were repeated three times.

Release of calcium

To study the release of calcium from intracellular stores during CPV infection, Fluo-4 Direct calcium assay kit was used (Invitrogen Life Technologies, CA, USA). Measurements were done according to manufacture's instructions. Shortly, cells grown on 96-well plates were synchronized and loaded with Fluo-4 for 60 min at 37°C. Loading solution was changed to DMEM and CPV or 100 nM thapsigargin (Invitrogen Life Technologies, CA, USA), an intracellular calcium releaser, were added to appropriate wells. Fluorescence intensity was measured with Victor X4 2030 Multilabel Reader (PerkinElmer, Waltham, MA, USA) at 30 min – 6 h after treatments using excitation wavelength 485 nm and emission was measured at 535 nm. Fluorescence intensity measurements were normalized in comparison with the mock infected control which was given the value 1. Experiment was repeated three times.

Activation of cell survival signalling

In order to examine the activation of cell survival signalling in CPV infected cells, phosphorylation of ERK1/2 was studied. NLFK cells were grown and infected with CPV as described above. As a positive control cells were incubated in the presence of 2 μM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) for 1 h (repeated twice). At indicated time points post infection cells were lysed in a 50 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate and 1:100 diluted protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 14 000× g and 4°C, protein concentration was determined with Bradford method (Bio Rad, Hercules, CA, USA). An amount of 50 μg of proteins were separated on SDS-PAGE gel and transferred to nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, NH). Blots were incubated with polyclonal antibody towards ERK1/2 or phosphorylated ERK1/2 (Cell Signaling Technology, Danvers, MA, USA) at a dilution 1:1000. Detection was performed with HRP-conjugated goat anti-rabbit antibody (Dako, Glostrup, Denmark) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Densitometry (ImageJ software, NIH, Bethesda, MD, USA) was utilized to measure the intensity of bands. Results were normalized to give a value of 1 for mock infected cells and CPV infected samples were compared to that. Experiments were repeated three times and results are shown as ratio of p-ERK to ERK.

Inhibition of ERK1/2 activation

To explore the importance of ERK1/2 activation in CPV infection, percentage of infected cells and depolarization of mitochondria were determined in the presence of the MEK1/2 specific inhibitor U0126 (Promega, Madison, WI, USA). U0126 was freshly dissolved at 10 mM in DMSO and added to the culture medium at final concentration of 20 μM for 30 min prior to virus infection and kept in the medium throughout the experiment. Samples were prepared for immunofluorescence microscopy to determine the infection percentage, for immunoblot analysis of ERK1/2 activation and for $\Delta\Psi$m analysis. Methods for immunofluorescence microscopy, immunoblot and $\Delta\Psi$m analysis were as previously described. Infection percentage was calculated at 24 h p.i.. Experiments with inhibitor were repeated three times.

Statistical testing

Student's t-test was used to identify statistically significant differences between mock and CPV infected samples. $p \leq 0.05$ was considered significant.

Results

CPV colocalized with mitochondria

To study the association between CPV and mitochondria, colocalization analysis based on confocal microscopy images was conducted. Results confirmed that cytoplasmic CPV colocalized with mitochondria from 2 h p.i. to 22 h p.i. (Fig. 1A). Colocalization of CPV and mitochondria, as manifested by white merge spots distributed in the cytoplasm, was observed at all time points used (Fig. 1A). Spots had an apparent perinuclear location at 10–14 h p.i..

A quantitative estimation of colocalization was obtained with BioImage XD software and is shown as a mean of 30 individual cells per time point (Fig. 1B). Percentage of cytoplasmic CPV

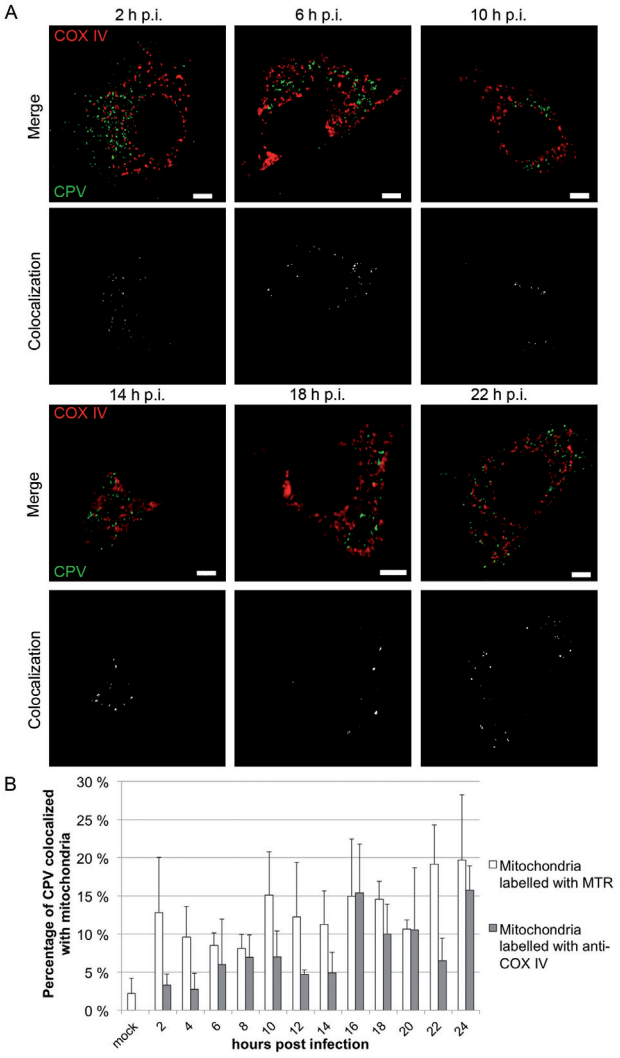

Figure 1. CPV colocalizes with mitochondria. (A) Confocal immunofluorescence images of CPV infected cells. Mitochondria were labelled with anti-COX IV antibody (red) and CPV with anti-capsid antibody (green). Nucleus has been cut out from the images due to really intense fluorescence from CPV at longer time points. Colocalization of CPV with mitochondria is shown in white in lower images. Bars 5 μm. (B) Percentage of colocalization of CPV with mitochondria. Colocalization analysis of confocal microscopy images was done with BioimageXD software. Mitochondria were labelled either with anti-COX IV antibody or with MitoTrackerRed (MTR). CPV was labelled with antibody recognizing intact capsids. Mock 24 h p.i. Results are shown as means from 30 cells from 3 independent experiments ± S.D.
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colocalizing (as defined by BioImage XD software) with mitochondria fluctuated during used time points. In MTR labelled cells the colocalization percentage was already 13% at 2 h p.i.. Then colocalization decreased to 9% at 4–8 h p.i.. At 10 h p.i. colocalization increased again to 15% and reached the highest level at 22–24 h p.i. with 19%. With anti-COX IV labelled cells

colocalization was low during entry (3–6% at 2–4 h p.i.), but percentage rose after 16 h p.i. and reached a highest level at 24 h p.i. (16%).

Infection with CPV caused mitochondrial damage

Immunoelectron microscopy was used to detect direct association of CPV with mitochondria and to study the ultrastructure of mitochondria in mock and CPV infected cells (Fig. 2). The preparation of samples within this technique is harsh. Unfortunately this can lead to some loss of the integrity of mitochondria as seen in the percentage of damaged mitochondria in the mock infected sample (6%). CPV located close to mitochondria at all of the studied time points (Fig. 2A). At 22 h p.i. virus label was seen in large plaques, not as rounded gold particles. These plaques were not located on the mitochondrial membrane as some label with the earlier time points. Some gold label was also seen occasionally inside the mitochondria at all time points. Damage to the mitochondrial morphology was seen in CPV infected cells. Infection seemed to cause disintegration of the membrane (as in Fig. 2A 6, 10, 18, 22 h p.i., 2B ii; marked with *). Additionally, mitochondrial membrane blebbing (Fig. 2B i, arrowhead) and disappearance of cristae (Fig. 2B ii, marked with C) was seen. At later time points starting 14 h p.i. damaged mitochondria were seen in autophagosome-like structures (Fig. 2B iii, marked with <). Percentage of damaged mitochondria was counted from 30 cells at each time point used and the mean is shown in Figure 2C. At 2 h p.i. the percentage of damaged mitochondria was 24%, but it decreased to 17% by 6 h p.i.. After this, the percentage rose and was the highest at 18 h p.i. when the percentage of damaged mitochondria reached 46%. These results demonstrated that CPV infection induced damage to the mitochondria.

Depolarization of mitochondrial membranes was associated with CPV infection

JC-1 dye was used to study the potential of mitochondrial membrane. CPV infected cells were labelled with this dye at different times post infection. Results demonstrated that mitochondria began to be depolarized at 2 h p.i. (Fig. 3) when the amount of depolarized mitochondria was 1.6 times the amount in mock infected cells. At 4 h p.i. $\Delta\Psi$m was restored to the level of mock infected cells. However, at 14 h p.i. depolarization of the mitochondrial membranes was seen again. At 18 h p.i. the amount of depolarized mitochondria was tripled when compared to mock infected cells. Valinomycin was used to induce depolarization of $\Delta\Psi$m in positive controls, and with this drug there were 27 ± 4.1 ($p\leq0.05$) times more depolarized mitochondria than in mock infected cells (data not shown). These results indicate that mitochondria are depolarized during CPV infection.

The ROS level increases during the early phases of CPV infection

Production of ROS during CPV infection was analysed with DCFDA. The intracellular level of ROS increased in the beginning of infection (Fig. 4). The amount of cells with increased ROS level at 2 h p.i. was 1.4 times the level in mock infected cells. Production of ROS continued to increase up to 6 h p.i., but returns to the level of mock infected cell from 8 h–22 hr p.i.. Thereafter the level of ROS increased again at 24 h p.i.. In STS treated apoptotic cells the level of ROS was 6.4 ± 1.5 ($p\leq0.05$) times the level in mock infected cells and in TBHP treated cells the factor was 6.0 ± 0.4 ($p\leq0.05$) (data not shown). These results demonstrate that mitochondria are affected during virus uptake, but cells are able to recover from the triggered oxidative stress.

Cytoplasmic calcium concentration remained unchanged in the beginning of infection

Fluorescent calcium indicator Fluo-4 was used to detect changes in cytoplasmic calcium concentration. Cells were loaded with Fluo-4 and treated with CPV or thapsigargin. Thapsigargin releases calcium from intracellular stores by inhibiting calcium-ATPases on endoplasmic reticulum. CPV infected and thapsigargin treated cells were compared to mock infected cells. Thapsigargin induced direct increase in cytoplasmic calcium when added to the cells as detected by increase in fluorescence (Fig. 5). In CPV infected cells only a minor change was observed in the fluorescence level indicating small fluctuation in cytoplasmic calcium concentration at 0.5–6 h p.i when compared to mock infected cells.

ERK1/2 was activated during a CPV infection

Due to the discovered changes in $\Delta\Psi$m at 2–4 h p.i., activation of cell survival signalling in the beginning of infection was studied. We used antibody towards phosphorylated ERK1/2 (p-ERK1/2) to investigate activation of ERK1/2 signalling. A representative result is shown in Fig. 6A. Ratio of band intensities of p-ERK1/2 to ERK1/2 increased already at 15 min p.i. (Fig. 6B). Activation was further enhanced to 30 min p.i.. Thereafter the activation started gradually to decline and by 4 h p.i. the ratio was below that of mock infected cells indicating a decrease in p-ERK. For cells incubated with PMA the ERK ratio was 9.8 ± 0.6 ($p\leq0.05$) (data not shown). When the activation of ERK1/2 was inhibited by U0126 the percentage of infection declined 20% when compared to infection without the drug (Fig. 6C). The used concentration of U0126 inhibited the activation of ERK1/2 efficiently as detected with immunoblotting (Fig. 6B). Inhibiting the ERK1/2 activation reduced the CPV induced depolarization of $\Delta\Psi$m at 2 h p.i. (Fig. 6D).

Discussion

Taking into account the important role of mitochondria in producing energy, defects in the mitochondrial functions reflect directly to the viability of the cell. Indeed, mitochondrial dysfunction is linked to pathologies of diseases among viral diseases [8,9,13,47]. As viruses are able to modulate mitochondrial function for their benefit this feature has evoked interest to be targeted for antiviral drugs. New antiviral drugs could inhibit viral changes in mitochondrial function or induce proapoptotic effect in virus-infected cells to inhibit viral replication [13,18,48]. To be able to design new antiviral drugs and to develop better intervention strategies, various virus-mitochondria interactions have to be carefully studied.

We have analysed the role of mitochondria in parvovirus pathology. Our data demonstrates that CPV localized to the cytoplasm near mitochondria at 2–24 h p.i. (Fig. 1). CPV associated with mitochondria through out the infection, but was only occasionally seen inside the mitochondria (Fig. 1 and 2). The percentage of CPV colocalized with mitochondria showed that at 2–24 h p.i. a fraction of CPV is associated with the mitochondria (Fig. 1). The amount of viruses associated with the mitochondria increased with longer time points after 16 h p.i.. By this time new viruses have been made [41,49] and the virus content of the cell has increased [41] probably affecting also the amount of association of CPV with the mitochondria. Colocalization percentages vary between the two used mitochondria label (Fig. 1B); MTR goes in the mitochondria, and labels the whole cell organelle as COX IV localizes at inner mitochondrial membrane. Different labelling of the mitochondria with these

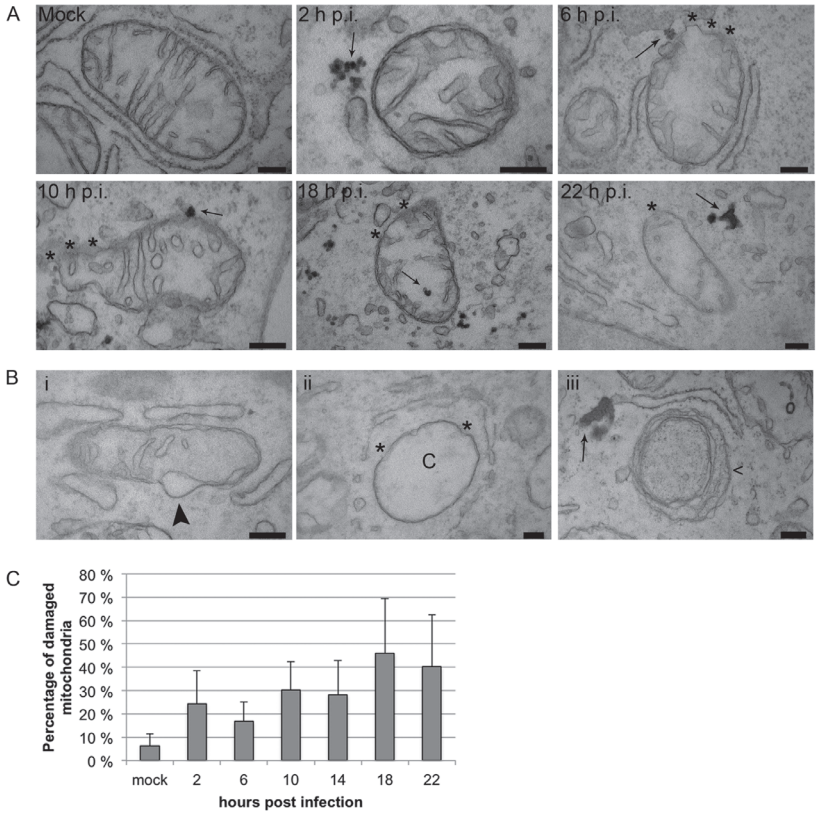

Figure 2. CPV infection influences the morphology of mitochondria. (A) Transmission electron microscopy pictures from mock and CPV infected cells at different time points post infection showing immunogold-labelled CPV to associate with mitochondria. (B) Following a CPV infection damage to the mitochondria can be observed. Images are from 22 h p.i. Infection leads to (i) membrane blebbing (arrowhead), (ii) damage to the membrane (*) and disappearance of cristae (C). (iii) Damaged mitochondria were seen inside autophagosome like structures. C) Percentage of damaged mitochondria was counted with transmission electron microscope from 30 cells from 3 independent experiments. Mock 2 and 22 h p.i. Results are shown as mean ± S.D and there is a statistically significant ($p \leq 0.05$) change between mock and CPV infected samples at all time points post infection. Bars 200 nm.
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dyes could account for the differences seen in colocalization percentages (Fig. 1B). Electron microscopy experiments showed that CPV was mainly associating with outer mitochondrial membrane (Fig. 2A) and not with inner mitochondrial membrane containing COX IV. Some of the increased mitochondrial damaged seen in the EM micrographs compared to the loss of membrane potential (Fig. 3) may also be due to the harsh EM technique. However as can be seen in Fig. 6 there is an activation of ERK that could counteract the loss of membrane potential at early stages of infection making this loss not a feature of lethal mitochondrial damage. To our knowledge, parvoviral proteins have not been earlier reported to associate with the mitochondria. However, localization of viral proteins in mitochondria has been reported for example for HCV [14], RSV [17], rotavirus [20], HIV [16] and herpes simplex virus [50].

During HCV infection, viral proteins target the mitochondria and cause enlargement of mitochondria and disappearance of cristae [14]. Vpr and gp160 proteins of HIV have been reported to induce deformation of cristae and disappearance of outer membrane [16,51]. EM studies presented that CPV infection induced similar morphological changes to the mitochondria (Fig. 2). These include rupture of mitochondrial membrane, disappearance of cristae and membrane blebbing. Damaged mitochondria started to collect after 10 h p.i.. Incoming virus (0–10 h p.i.) affected the mitochondrial structure only transiently, but expression of NS1 (after 10 h p.i. [41,49]) and production of new viruses further damaged the mitochondrial structure.

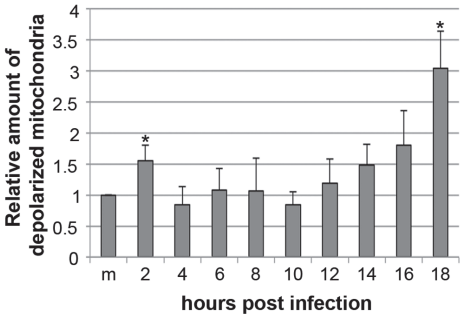

Figure 3. Biphasic loss of mitochondrial membrane potential during CPV infection. Flow cytometry of JC-1 labelled cells was used to detect depolarization of mitochondrial membrane potential during CPV infection. Results are shown as mean of relative amount of cells showing mitochondrial depolarization from three independent repeats (m, mock infected 18 h p.i.). * The difference is statistically significant ($p \leq 0.05$) between marked time point and mock infected cells.
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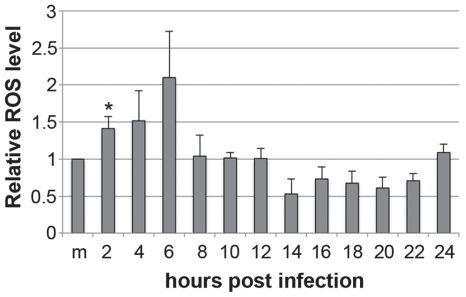

Figure 4. Production of ROS during CPV infection. The level of ROS was determined by loading the cells with DCFDA and analyzed by flow cytometry. Results are shown as mean of relative amount of cells showing production of ROS from three independent repeats (m, mock infected 24 h p.i.). * The difference is statistically significant ($p \leq 0.05$) between marked time point and mock infected cells.
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Figure 2C shows less damage to the mitochondria at 22 h p.i. than at 18 h p.i.. This decline depends on the decreased cell amount as infected cells start to die and detach from the dish at 22 h p.i.. Incubation of purified mitochondria from HeLa cells with a parvovirus minute virus of mice (MVM) did not induce damage to mitochondrial membranes [52]. Furthermore, microinjected MVM did not damage mitochondria in *Xenopus* oocytes although MVM was able to rupture nuclear envelope [52]. The reported results differ from our results in that we demonstrate that CPV damages the mitochondria during an infection. However, in experiments with MVM [52] there was no viral replication and in our experiments the damaged mitochondria accumulated after the beginning of viral replication (Fig. 2C).

Autophagocytosis is a normal way for cells to remove damaged and malfunctioning mitochondria to favour cell surviving [53,54]. Double membrane vesicle is a characteristic of autophagosomes [55,56]. EM studies revealed that damaged mitochondria were seen inside structures resembling autophagosomes (Fig. 2B, iii) at later time points, starting from 14 h p.i.. Infection of UT7/Epo-S1 cells with human parvovirus B19 (B19V) has been reported to induce autophagocytosis [57]. In B19V infection autophagosomes contained degraded mitochondria and the autophagocytosis was connected to the survival of infected cells from apoptosis. Similarly, in our studies the autophagosome-like structures contained damaged mitochondria. However, the implication of observed autophagocytosis remains to be elucidated.

The mitochondrial function is dependent on intact mitochondria. One indicator of mitochondrial health is $\Delta\Psi$m the dissipation of which is connected to the intrinsic mitochondrial apoptotic pathway [58]. Our experiments revealed that mitochondria were depolarized shortly in the beginning of infection (Fig. 3). However, the final depolarization of larger population of mitochondria appeared later, about 14–16 h p.i. (Fig. 3), by the time that viral replication has started as detected by the NS1 expression [41]. Dissipation of $\Delta\Psi$m indicates permeabilization of mitochondrial inner membrane that leads to cell death [3,59,60]. AAV-2, belonging to the adeno-associated parvoviruses, has been reported to sensitize cells to apoptosis by targeting mitochondria [61]. In combination with cisplatin AAV-2 induced depolarization of $\Delta\Psi$m that was not detected with AAV-2 or cisplatin alone. However, the mechanism involved in sensitizing mitochondria has not been studied. Increased production of ROS is often associated with mitochondrial, proteins, lipids and DNA damage [62]. Additionally ROS play a role in induction of cell death as reported for rat parvovirus H-1 [63]. The level of ROS increased in our studies during the early phases of infection (Fig. 4). However, at 8 h p.i. the level of ROS had declined to the level of mock infected cells indicating that the production of ROS only at later time points (post-24 h) could be a contributing element to apoptosis. The final factor to induce cell death could also be mediated through a synergy of other pathways, such as DNA damage and caspase activation [41,64,65]. B19V induces apoptosis through mitochondrial cell death pathway in non-permissive cells [66]. The trigger for the intrinsic apoptotic route is from NS1 induced DNA damage [64,65]. Our results suggest that mitochondrial pathway is involved in CPV induced cell death, possibly by a

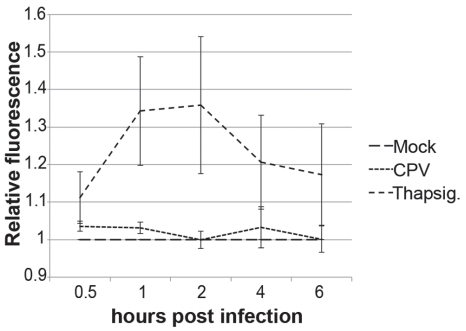

Figure 5. Calcium is not released to the cytoplasm during the entry phase of infection. Release of calcium from intracellular stores, like the mitochondria, was studied with Fluo-4 dye. Cells were loaded with Fluo-4 and infected with CPV. At indicated time points p.i. fluorescence intensity was measured. Thapsigargin was used as a positive control. Results are means from 3 repeat ± S.D.
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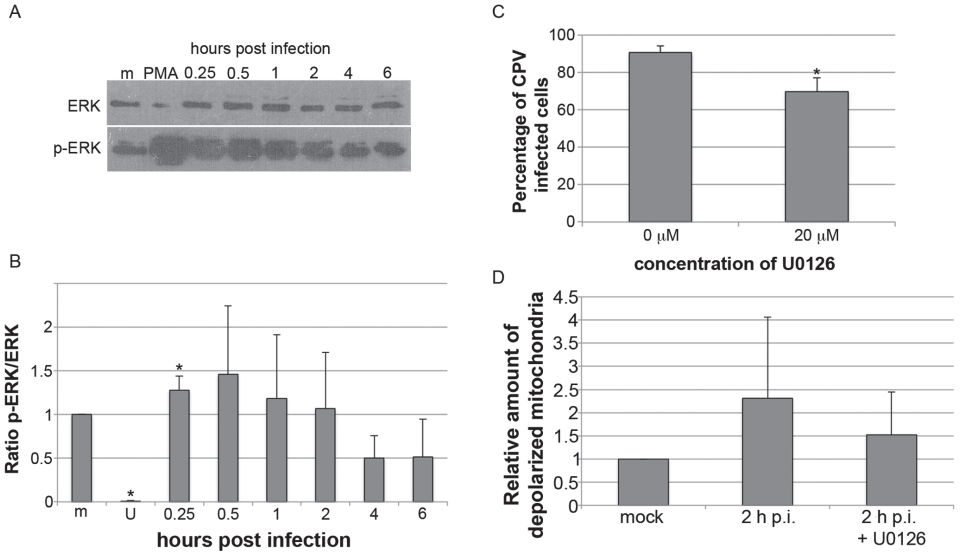

Figure 6. ERK1/2 signalling is activated early in CPV infection. (A) Activation of ERK1/2 was analyzed based on phosphorylation of ERK1/2 (p-ERK1/2). Cell lysates were collected at indicated time points post infection and immunoblotting with antibody towards ERK or p-ERK1/2 was performed. (B) Intensity of band signals were determined with densitometry (ImageJ software) and ratio of p-ERK to ERK was calculated. * The difference is statistically significant ($p \leq 0.05$) between marked time point and mock infected cells (m, mock infected 6 h p.i.; U, U0126). (C) CPV infection was carried out in the presence of 20 μM U0126 that inhibits the activation of ERK1/2. At 24 h p.i. cells were fixed and CPV infected cells were visualized with anti-capsid antibody. Infection percentage was determined with immunofluorescence microscopy. The observed decrease in infection percentage was statistically significant ($p \leq 0.05$). (D) Cells were infected with CPV or mock infected with or without 20 μM U0126 and JC-1 was used to detect the depolarization of mitochondrial membrane potential. Results are shown as mean of relative amount of cells showing mitochondrial depolarization from three repeats.
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mechanism similar to that observed for B19V [65]. The direct contact observed between CPV and mitochondria in the beginning of infection (Fig. 1 and 2, 0–10 h p.i.) did not launch an apoptotic process, but was triggered after initiation of viral replication as detected by NS1 expression [41].

Parvoviruses harbour a phospholipase A2 (PLA2) activity in their VP1 protein [67]. Expression of B19V VP1 protein has been reported to increase the cytoplasmic calcium concentration by activating I_{CRAC} channels on plasma membrane in endothelial cells [68]. Additionally, cytoplasmic calcium concentration controls $\Delta\Psi$m [1] and infection of viruses, involving poliovirus, rotavirus, hepatitis B virus and HIV, induces an increase in cytoplasmic calcium concentration that is connected to the depolarization of $\Delta\Psi$m and initiation of apoptosis [69–72]. Due to the observed change in $\Delta\Psi$m at 2 h p.i. (Fig. 3) we analysed the changes of the intracellular calcium concentration. However, the calcium concentration was stationary (Fig. 5).

When the colocalization of CPV with mitochondria was studied by labelling mitochondria with MTR the percentage of colocalization was already 13% at 2 h p.i. (Fig. 1). At the same time damage to mitochondria and change in the mitochondrial homeostasis could be seen as some mitochondria lost their $\Delta\Psi$m and the level of intracellular ROS increased (Fig. 2C, 3 and 4). The mitochondria can sense intracellular stress through different signalling cascades and responds to those in order to get back to homeostasis [3]. In CPV infected cells the homeostasis was gained back at 4 h p.i. as the $\Delta\Psi$m was normalized to the level of mock infected cells (Fig. 3). As ERK1/2 signalling is involved in inhibition of apoptosis at the mitochondrial level [22] we tested for ERK1/2 activation, a cell survival signal, in the very early phases of a CPV infection (Fig. 6). It is demonstrated that indeed ERK1/2 was activated, as could be detected with an increase in p-ERK (Fig. 6A, B), at 15 min – 2 h p.i.. Additionally, inhibition of ERK1/2 activation partially prevented depolarization of $\Delta\Psi$m at 2 h p.i. (Fig. 6D). ERK1/2 activated signalling promotes cell survival and prevents the stress situation evoked by the cell due to invading viruses. A virus benefits from this kind of signalling by receiving more time for viral replication as seen with other viruses like coxsackievirus B3, enterovirus 71 and influenza virus [24,25,28]. In parvovirus studies, inhibition of ERK1/2 activation by U0126 decreased the ability of CPV to replicate in host cells (Fig. 6C) indicating the importance of ERK1/2 activation. In another study, stimulation of macrophages with B19V VP1 protein, that contains PLA2 domain, has been reported to also activate ERK1/2 signalling [73]. In addition, activation of ERK1/2 signalling decreased during a B19V infection in $CD36^+$ erythroid progenitor cells (EPC) cultured under hypoxia [74]. The B19V was shown to replicate more efficiently under hypoxia and in these culture conditions one important factor favourable for B19V replication was the decrease of ERK1/2 activation. In $CD36^+$ EPCs the decline in activated ERK1/2 was connected to the lower level of EPC differentiation [74]. Here we report reverse

action of ERK1/2 signalling during parvovirus infection. However, for both CPV and B19V the change in ERK signalling accounts for the purpose of ensuring the production of progeny. Other survival pathways, like the PI3K/AKT pathway utilized by rotavirus [20] or pathways inhibiting apoptosis [75], are possibly having a role during CPV infection.

Parvoviral NS1 protein is known for its cytotoxic nature [35,36,42,63–65]. Changes of the mitochondria displayed in this study occurred concomitantly with the earlier reported schedule of NS1 expression [41,49]. We have earlier shown that caspase 9, the caspase involved in intrinsic apoptotic pathway, is activated already 6 h p.i.. Significant activation of caspase 3 and damage to nuclear DNA can be detected at 12 h p.i. [41]. By 6 h p.i. viral replication has not started yet, consequently this activation is likely to happen through cellular signalling pathways. This is supported by the results of this study as mitochondrial structures were mainly intact at 6 h p.i. (Fig. 2 and 3). Increase in damaged mitochondria (Fig. 2 and 3) and DNA [41] could be seen after time of onset of viral protein expression, especially that of NS1, after 12 h p.i.. Even though CPV was shown to associate with the mitochondria directly, this contact does not seem to induce cell death. Early damage to mitochondria at 2 h p.i. (Fig. 3), production of ROS (Fig. 4) and caspase activation at 6 h p.i. [41] indicate that virus infection is sensed by the host and it reacts not by being pushed to intrinsic cell death but to cell survival. As seen in Fig. 3, the mitochondrial membrane potentials are of the same levels as with mock infected cell between 4–12 h p.i.. This may be due to the long lasting effect of the activated ERK signalling cascade. We speculate that the activated ERK1/2 signalling is involved in regaining the mitochondrial homeostasis along with other survival signalling pathways and apoptotic changes are starting to collect only after 14 h p.i. (Fig. 3), after beginning of viral replication and NS1 expression. Taken together, there is association of CPV and the mitochondria at very early time points and onwards, but this is not the initial signal to push the cell into apoptosis. ERK1/2 signalling is activated in the beginning of infection to ensure cellular viability. After beginning of viral replication the involvement of mitochondria in the cell death can be seen as indicated by mitochondrial damage (Fig. 2 and 3). Results obtained with this study are useful for understanding parvoviral pathology and also in more general scale provide information about virus-mitochondria association.

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Author Contributions

Conceived and designed the experiments: JN MV LG. Performed the experiments: JN. Analyzed the data: JN LG. Contributed reagents/materials/analysis tools: JN MV LG. Wrote the paper: JN MV LG.

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III

LATE STEPS OF PARVOVIRAL INFECTION INDUCE CHANGES IN CELL MORPHOLOGY

by

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