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Parvovirus infection-induced cell death and cell cycle arrest

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Abstract

The cytopathic effects induced during parvovirus infection have been widely documented. Parvovirus infection-induced cell death is often directly associated with disease outcomes (e.g., anemia resulting from loss of erythroid progenitors during parvovirus B19 infection). Apoptosis is the major form of cell death induced by parvovirus infection. However, nonapoptotic cell death, namely necrosis, has also been reported during infection of the minute virus of mice, parvovirus H-1 and bovine parvovirus. Recent studies have revealed multiple mechanisms underlying the cell death during parvovirus infection. These mechanisms vary in different parvoviruses, although the large nonstructural protein (NS)1 and the small NS proteins (e.g., the 11 kDa of parvovirus B19), as well as replication of the viral genome, are responsible for causing infection-induced cell death. Cell cycle arrest is also common, and contributes to the cytopathic effects induced during parvovirus infection. While viral NS proteins have been indicated to induce cell cycle arrest, increasing evidence suggests that a cellular DNA damage response triggered by an invading single-stranded parvoviral genome is the major inducer of cell cycle arrest in parvovirus-infected cells. Apparently, in response to infection, cell death and cell cycle arrest of parvovirus-infected cells are beneficial to the viral cell lifecycle (e.g., viral DNA replication and virus egress). In this article, we will discuss recent advances in the understanding of the mechanisms underlying parvovirus infection-induced cell death and cell cycle arrest.

Keywords

cell cycle arrest; cell death; DNA damage response; Parvovirus

Parvoviruses have a nonenveloped icosahedral virion with a diameter of approximately 20–25 nm, which encapsidates a linear, ssDNA genome of an average size of 5000 bases [1]. The family *Parvoviridae* contains two subfamilies: *Parvovirinae* and *Densovirinae*. The latter infects only invertebrates, and will not be the subject of this article. The family *Parvovirinae* is composed of five genera: *Amdovirus*, *Bocavirus*, *Dependovirus*, *Erythrovirus* and *Parvovirus* [2]. Adeno-associated viruses (AAVs) in the genus *Dependovirus* require helper viruses (e.g., adeno-viruses) for productive infection [3]. All other members of *Parvovirinae* do not require helper virus, and are called autonomous parvoviruses. In addition, three genotypes of human parvovirus 4 have been discovered and proposed as the sixth genus in *Parvovirinae*, although little is known about their biology and

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pathogenesis [4–7]. In this article, the term ‘parvovirus’ refers to viruses in the subfamily of *Parvovirinae*.

Disease outcomes of parvovirus infection vary among members of *Parvovirinae*. Human parvovirus B19 (B19V) is the only member that has been confirmed to be pathogenic to humans [8]. B19V infection is the cause of an increasing list of diseases, including fifth disease in children, transient aplastic crisis in patients with chronic hemolytic anemia (e.g., sickle cell disease patients), pure red-cell aplasia in persistent infection in immunocompromised patients, and hydrops fetalis in pregnant women [8–13]. Human bocavirus (HBoV), which was identified in 2005, has been linked to lower respiratory tract infections in children [14–16], and was confirmed to be associated with severe pneumonia in children coinfected with other respiratory viruses [17,18]. Of animal parvoviruses, Aleutian mink disease virus (AMDV), the only member of the genus *Amdovirus*, causes the main symptoms of gastroenteritis, aplastic anemia and lymphopenia [19]. In the genus *Bocavirus*, minute virus of canines (MVC) causes respiratory diseases with breathing difficulty, enteritis with severe diarrhea, spontaneous abortion of fetuses and the death of newborn pups [20–22]. Similar enteritis was also seen in bovine parvovirus (BPV)-infected calves [23,24]. In the genus *Parvovirus*, infection of feline parvovirus and canine parvovirus cause gastrointestinal disease (e.g., enteritis) [25–27]. Porcine parvovirus was identified as the cause of stillborn and mummified piglets [28].

The cytopathic effects (CPEs) induced during parvovirus infection have been widely documented. The disease outcomes of parvovirus infection are often the result of CPEs (e.g., cell death of B19V-infected erythroid progenitor cells causes anemia and nonimmune hydrops fetalis) [29,30]. Cell death induced during parvovirus infection appears to be mediated by either apoptosis or necrosis (nonapoptotic cell death), whereas cell cycle arrest is often observed as one of the early responses of host cells to parvovirus infection.

Apoptosis and necrosis are two types of cell death that are tightly controlled by distinct programmed signaling cascades. Apoptosis is defined mechanistically into two major pathways (i.e., extrinsic and intrinsic) both involving sequential activation of caspases. The extrinsic pathway is activated by the ligation of ‘death’ ligands, such as Fas ligand and TNF-α to ‘death’ receptors, followed by activation of caspase-8/caspase-10 and a cascade of caspase activation, which eventually leads to apoptosis [31]. The mitochondrion-mediated (intrinsic) pathway is activated by multidomain Bcl-2 family proteins, notably Bax and Bak [32,33]. This pathway causes mitochondrial outer-membrane permeabilization, resulting in the release of cytochrome c, Smac and Omi into cytosol, where they work with apoptotic protease-activating factor-1 to form the apoptosome complex, followed by the activation of capase-9 and, eventually, apoptosis [34]. Caspase-3, -6 and -7 are the downstream executor caspases [35]. In addition, the crosstalk between the extrinsic and intrinsic pathways has been well established (e.g., activated caspase-8/-10 cleaves Bid to tBid, which, in turn, activates Bax/Bak) [36,37].

Necrosis, catastrophic cell death, is characterized by organelle swelling, mitochondrial membrane dysfunction, massive oxidative stress and rapid plasma membrane permeabilization [38]. Previously, necrosis was thought to be unregulated cell death; however, recently, a number of regulated nonapoptotic cell death pathways have been attributed to necrosis [39].

Cell cycle checkpoints are cellular mechanisms that ensure each and every step of the replication cycle takes place accurately and precisely at the right time. Each cell cycle checkpoint is controlled by different cyclins and cyclin-dependent kinases (CDKs) [40,41]. An aberrant cellular environment change or exogenous assault results in cell cycle

checkpoint arrest, which provides time for cells to recover and return to their normal status before proceeding to the next step of the cell cycle [42].

Human parvovirus B19

B19V infection has an exclusive tropism for both $CD36^+$ human erythroid progenitors and erythroblasts of the human bone marrow and fetal liver [43–46]. Clinical symptoms, as seen in anemia and nonimmune hydrops fetalis, are the direct result of the destruction of erythroid progenitors in bone marrow and fetal tissues by virus infection [29,30]. Tissue samples from hydrops fetalis patients infected with B19V have been found to have characteristics of apoptosis [46]. Fetal erythroid progenitors infected by B19V reveal ultrastructural features of apoptotic cell death [43].

In vitro studies of B19V infection-induced CPE have long been hampered by the lack of a permissive culture system. The first productive *in vitro* culture of B19V, reported in 1987, used bone marrow cells directly collected from a sickle cell disease patient [47]. The establishment of UT7/Epo-S1, a subclone of megakary-oblastic cell line UT7/Epo [48], had allowed the study of the role of viral non-structural (NS) proteins in B19V infection-induced cell death [49]. However, the UT7/Epo-S1 cells are, at best, semipermissive, as only 5–10% of the viral antigen-expressing cells can be detected during B19V infection, let alone the production of infectious virion [50,51]. *Ex vivo*-expanded erythroid progenitor cells have made it possible to carefully examine cell death induced during B19V infection [51–53].

B19V infection of human bone marrow has been shown to inhibit both burst-forming erythroid units and colony-forming erythroid units, resulting in an arrest of erythropoiesis [44,45]. Previously, the cytotoxicity of B19V infection was believed to be a direct function of NS1 expression [54]. The molecular mechanisms leading to apoptosis during B19V infection of primary erythroid progenitor cells was investigated in parallel with inducible stable NS1 expression in UT7/Epo cells [53]. Both B19V infection in erythroid progenitor cells and NS1-induced apoptosis were inhibited by caspase-3, -6 and -8 inhibitors. Substantial caspase -3, -6 and -8 activities were induced by NS1 expression in UT7/Epo cells [53]. The caspase-mediated apoptosis by NS1 expression in UT7/Epo cells was evidenced by cell morphology, genomic DNA fragmentation [49], and the fact that stable expression of Bcl-2, an antiapoptotic protein, resulted in near-total protection from cell death in response to NS1 induction [49]. Caspase-8, which mediates the extrinsic apoptotic pathway, was activated during B19V- and NS1-induced apoptosis. Consequently, B19V-infected erythroid progenitors and NS1-expressing UT7/Epo cells were sensitized to TNF-α-induced apoptosis [53]. Moreover, the ceramide level was enhanced by B19 infection and NS1 expression. Therefore, a connection between the apoptotic pathways activated by TNF-α and NS1 in B19V-infected human erythroid progenitor cells was proposed.

B19V NS1 has been shown to induce apoptosis in nonpermissive cell lines as well [49,55,56]. In addition, expression of NS1 transgene in mice, under the erythroid lineage-specific GATA-1 promoter, has been demonstrated to be embryonic lethal as a result of fatal anemia [57]. In nonpermissive liver-derived cell types, both B19V inoculation and NS1 expression were shown to activate caspase-3 and -9, but not caspase-8 [55,58]. Treatment of transfected cells with inhibitors of caspase-3 or -9 significantly inhibited apoptosis. Neutralization of TNF-α or Fas ligand had no effect on apoptosis induced in liver cells. In monkey epithelial cells, COS-7, expression of NS1 induced an increased level of apoptosis [56]. Consistent with the results from liver cells, the increased expression of p53, the proapoptotic Bcl-2 members Bax and Bad, and activation of caspase-3 and caspase-9, but not the activation of caspase-8 or Fas, were detected in the NS1-transfected cells. Furthermore, a p53 inhibitor abolished activation of caspase-9, and apoptosis was

significantly diminished by the caspase-9 inhibitor [56]. Thus, NS1 appears to induce intrinsic mitochondrion-mediated apoptosis in nonpermissive cells.

The B19V NS1 protein is a multifunctional protein during the virus lifecycle [59–63]. Loss-of-function mutations engineered into the nucleoside triphosphate (NTP)-binding domain of NS1 significantly rescued cells from NS1-induced apoptosis, without having any effect on NS1-induced activation of IL-6 gene expression, which is mediated by NF-κB [49]. Furthermore, using pentoxifylline, an inhibitor of NF-κB activation, NF-κB-mediated IL-6 activation by NS1 was shown to be uncoupled from the apoptotic pathway. Thus, induction of apoptosis by NS1, at least in UT-7/Epo-S1 cells, is a separate function from transactivation. The loss-of-function mutation of the NTP-binding site of NS1 also significantly decreased apoptosis in HepG2 cells [55]. Therefore, the NTP-binding motif of B19V NS1 is responsible for NS1-induced apoptosis in both B19V semipermissive and nonpermissive cell lines.

Overall, B19V NS1-induced apoptosis is characterized by caspase activation and DNA fragmentation. The extrinsic pathway, and TNF-α-induced activation of this pathway in particular, is suggested to be responsible for NS1-induced apoptosis in permissive cells, while in non-permissive cells, the mitochondrion-mediated intrinsic pathway is activated. Nevertheless, the NTP-binding motif of NS1 is the only domain identified to be responsible for inducing apoptosis in both semipermissive and nonpermissive cells. Based on this evidence, NS1 has long been thought to be the only viral protein of B19V that induces apoptosis during B19V infection. However, the NS1-induced apoptosis could be exaggerated in both nonpermissive and semipermissive cells, as the kinetics of NS1 expression do not correlate with those of induction of apoptosis during B19V infection [64].

Using $CD36^+$ erythroid progenitor cells *ex vivo* expanded from primary $CD34^+$ hematopoietic stem cells [51], we have identified that the B19V small NS protein 11 kDa as a novel inducer of apoptosis during B19V infection, which is mediated through caspase-10 activation [52]. The 11 kDa protein is expressed dominantly in the cytoplasm during B19V infection, at a level at least 100-times greater than NS1, which is solely expressed in the nucleus during the course of infection in $CD36^+$ erythroid progenitor cells. By further knockdown of 11 kDa expression using antisense oligos, we confirmed that the 11 kDa protein plays a key role in killing $CD36^+$ erythroid progenitor cells during B19V infection. Interestingly, 11 kDa was reported to interact with growth factor receptor-bound protein 2 (Grb2) via the SH3 domain binding motif *in vitro* [65]. The mechanism underlying B19V 11-kDa-induced apoptosis, and especially the potential significance of the 11-kDa–Grb2 interaction, warrants further investigation.

The G2/M checkpoint arrest induced during B19V infection has been reported in both primary $CD36^+$ erythroid progenitor cells [53] and the cell line UT7/Epo-S1 [48]. In UT7/Epo-S1 cells, B19V infection induced an accumulation of cyclin A, cyclin B1 and phosphorylated cdc2, and was accompanied by an upregulation in the kinase activity of the cdc2–cyclin B1 complex, which is consistent with G2/M checkpoint arrest [48]. In agreement, degradation of nuclear lamina and phosphorylation of histone H3 and H1, markers of M-phase, were not seen in B19V-infected cells. Moreover, accumulation of cyclin B1 was persistently detected in the cytoplasm, but not in the nucleus, suggesting that B19V infection contributes to the suppression of the nuclear import of cyclin B1.

G0/G1 arrest was also demonstrated in B19V-infected UT7/Epo-S1 cells with application of the mitotic inhibitor paclitaxel [66]. NS1-expressing UT7/Epo-S1 and 293T cells were shown to undergo cell cycle arrest at the G0/G1 rather than the G2/M checkpoint. NS1 expression significantly increased $p21^{WAF1}$ expression, a CDK inhibitor that induces G0/G1

arrest. In addition, G0/G1 arrest mediated by NS1 was proposed to be a prerequisite for the apoptosis of erythroid progenitor cells during B19 virus infection. In monkey epithelial cells, COS-7, expression of NS1 also induced G0/G1 checkpoint arrest, accompanied with an increased level of apoptosis [56]. The expression of p53 and its downstream cell cycle kinase inhibitors, $p16^{INK4}$ and $p21^{WAF1}$, were upregulated in NS1-transfected cells. Given the requirement of the S-phase for the replication of parvoviruses, the role of NS1-induced G0/G1 arrest in the B19V cell lifecycle needs to be addressed.

G2/M arrest appears to be important for B19V infection, as inhibition of G2/M arrest by caffeine significantly decreased the expression of NS1 [66]. However, the mechanism underlying B19V infection-induced G2/M checkpoint arrest, especially which viral components are involved, remains elusive. In our recent work, we found that cell cycle arrest occurred as one of the earliest events during infection [64] [Chen AY, Qiu J, Unpublished Data]. While UV-inactivated B19V did not express NS1, it induced G2/M arrest at a level similar to that induced by the infectious virus [66], indicating that the viral genome might play an important role in the cell cycle arrest. Expression of B19V viral proteins (NS1, VP1, VP2, 11 kDa and 7.5 kDa) in UT7/Epo-S1 cells by transfection did not change the cell cycle pattern. However, transfection of a viral sequence containing half of the left-hand inverted terminal repeat did partially reproduce G2/M arrest [Chen AY, Qiu J, Unpublished Data]. Only recently, a CpG oligode-oxynucleotide 2006, with consensus sequence located in the P6-promoter region of B19V genome (5′-GTTTTGT-3′), directly inhibited the growth of burst-forming erythroid cells, resulting in the accumulation of cells in the S and G2/M-phases, and increasing cell size and frequency of apoptotic cells [67]. Therefore, the B19V viral genome seems to play an important role in arresting the cell cycle at G2/M.

Overall, apoptosis is the programmed cell death pathway underlying B19V infection of erythroid progenitors. Viral proteins, NS1 and 11 kDa in particular, are responsible for promoting the apoptotic process; however, the 11-kDa protein is the major apoptotic inducer, considering it is expressed at a level 100-times greater than that of NS1 during B19V infection. Other to-be-identified factors, such as the viral genome and the 7.5-kDa small NS protein, may also work in concert to ensure cell death outcome. G2/M checkpoint arrest by the B19V genome occurs early during infection, and may contribute to the CPE during the early phase of B19V infection. Although the underlying mechanism is largely unknown, a viral genome-induced DNA damage response-mediated pathway is likely involved.

Amdovirus

Aleutian mink disease virus was the first parvovirus known to utilize caspase activity to facilitate its replication [19,68]. AMDV infection has been shown to induce caspase activation, and results in apoptotic cell death [19]. Pretreatment of infected cells with caspase-3 or broad- spectrum caspase inhibitors not only prevented apoptosis but also caused a reduction (by 2 $\log_{10}$) in production of progeny infectious viruses compared with untreated controls. Thus, permissive replication of AMDV *in vitro* in Crandel feline kidney (CrFK) cells depends upon activation of caspase-3. Furthermore, active caspase was confirmed to be required to specifically cleave NS1 protein at two sites, aa227 (INTD↓S) and aa285 (DQTD↓S), and the cleavage products were crucial for the replication of the AMDV genome [68]. Importantly, the NS1 products could be identified in AMDV-infected cells but were not present in infected cells pretreated with caspase inhibitors. When the two caspase cleavage sites were mutated (D to E) in an infectious clone, replication of the clones containing either of these mutations was reduced by 3–4 $\log_{10}$-fold compared with that of the wild-type clone, and the clone with both mutations was replication defective.

Mechanistically, immunofluorescence staining demonstrated that cleavage was required for nuclear localization of NS1.

Our recent work further demonstrated the critical role of caspase activation during productive infection of AMDV. We proved that expression of the viral capsid proteins alone can activate caspases, including caspase-10, which may serve as an initiator [69]. *In vitro* caspase cleavage assays showed that the effector caspase-7 further specifically cleaved the capsid protein VP2 at D420. AMDV mutants that are resistant to caspase-mediated capsid cleavage increased virus production by approximately three- to five-fold in CrFK cells. Thus, caspase-mediated specific cleavage of capsid proteins might have a role in regulating persistent infection of AMDV in animals. Collectively, caspase activation plays multiple roles in infection of AMDV, through both promoting replication of the viral genome and limiting capsid production.

S-phase cell cycle was shown to be required for the expression of the AMDV NS1 protein [70]. Cells that progressed through S-phase showed a characteristic binary pattern of cell cycle disturbance caused by AMDV infection. While a small portion of NS1-expressing cells escaped the G2/M checkpoint arrest and progressed to the G0/G1-phase, the majority were arrested at a postmitotic phase, with DNA content higher than 4N. Intriguingly, active DNA synthesis was detected in cells arrested at the postmitotic phase, which implies the potential role of postmitotic phase arrest for AMDV genome replication. Nevertheless, the nature of the postmitotic DNA content in arrested cells, whether of host or viral origin, is not clear. Which viral components are responsible for the cell cycle arrest still remains to be determined.

In conclusion, apoptosis and, in particular, activated caspases, regulate AMDV infection by specific cleavage of both NS1 and capsid proteins. Infected cells with apparent cell cycle arrest still support AMDV replication.

Bocavirus

The importance of bocavirus infection was raised with the emergence of HBoV. HBoV infection has been suggested to be associated with pneumonia and, possibly, gastroenteritis, mainly in children [14–16]. As the *in vitro* infection of HBoV is far from efficient [71], it is currently difficult to reproduce HBoV infection in routine laboratory settings. The genetic map of HBoV1 was recently described, and confirmed to be very close to that of MVC [71,72]. Therefore, the study of bocavirus-induced CPE is basically carried out with the two other members of bocavirus, namely MVC and BPV [73,74].

Significant CPE has been observed during *in vitro* infection of Walter Reed canine cell/3873D (WRD) cells by MVC [75,76]. We have established an MVC-infectious clone that produces the infectious virus efficiently [76]. We took advantage of the MVC *in vitro* infection system as a model for the bocavirus genus, and explored the molecular mechanism underlying the cell death induced during bocavirus infection [74]. We found that MVC infection triggered replication-dependent, mitochondrion-mediated apoptosis, which can be blocked by a pan-caspase inhibitor. Moreover, the level of cell death correlated closely with the level of MVC replication. Expression of viral proteins individually, or in combination, failed to induce cell death in transfected cells.

Along with apoptosis, we also observed a progressive cell cycle arrest of infected WRD cells [74]. At approximately 18 h after infection with MVC, NS1-expressing WRD cells showed a single widened cell-cycle peak, with a plateau at S-phase, which progressed into prolonged G2/M arrest. The DNA content of NS1-expressing WRD cells did increase to slightly higher than 4N at G2/M arrest. We believe this finding was the result of the

replicated viral genome, which was quantified to be approximately an eighth the size of the human genome during active replication at 48 h postinfection. UV-inactivated MVC also induced cell cycle arrest at the G2/M, but not S-phase. The cell cycle was gradually resolved without inducing a significant level of cell death. Moreover, when transfected with a panel of mutants of the MVC infectious clone, MVC viral protein expression did not induce either apoptosis or cell cycle arrest, while the genome alone induced G2/M arrest, even when half of the left terminal-repeat structure was deleted. The S-phase plateau, which has not been seen in other members of *Parvovirinae*, appeared only when active MVC replication took place.

By contrast, CPE induced during BPV infection was shown to be mediated by necrosis rather than apoptosis [77]. With the use of embryonic bovine tracheal cells, BPV infection did not cause alterations in nuclear morphology, membrane changes, apoptotic body formation, membrane phosphatidylserine inversions, caspase activation or cellular DNA fragmentation. On the other hand, at the end of the virus replication cycle, infected cells released viral hemagglutinin and infectious virus particles, as expected from cell membrane failure. Moreover, the infected cells released lactate dehydrogenase, a marker of necrosis, which directly correlated with virus production. Furthermore, assessment of mitochondrial dehydrogenase activity was consistent with cell death by necrosis.

Collectively, bocavirus infection induces either apoptosis or necrosis, depending on the type of virus. Cell cycle manipulation by bocavirus is unique, in that the S-phase plateau in early infection progresses into G2/M arrest at a later stage.

Dependovirus

Adeno-associated viruses have not been associated with any disease in humans [3]. Recombinant AAV has been favorably emphasized as a vector for human gene therapy [78,79]. However, there are several reports of AAV infection in human maladies (e.g., male infertility [80] and complications of pregnancy [81–83]). A series of reports emerged recently that investigated cell death induced by AAV infection, as well as the underlying mechanism [84–86]. Identification of the AAV-mediated killing of cells lacking p53 activity has opened up the exploration of the oncolytic potential of AAV [86]. The p53–p21-phosphorylated retinoblastoma protein (pRb) pathway protects normal cells from AAV-induced apoptosis [87]. The lack of this pathway, by loss or mutation of p53, p21 or pRb, sensitized cells, particularly tumor cells, to AAV infection-induced apoptosis. Moreover, DNA damage effector kinase Chk1 was also suggested to be the mediator of apoptotic cell death induced during AAV infection of p53-deficient cells [88]. In addition, it was reported that the NS protein Rep78 of AAV2, independent of p53, induced apoptosis with activation of a caspase cascade [89].

While apoptotic cell death was attributed to the activation of caspase cascade, emphasis was placed on mechanistically linking the DNA damage response induced during AAV infection to cell cycle regulation and p53-independent cell death. G2/M arrest was observed during AAV infection, but intriguingly not in $ATM^{-/-}$ cells [86]. S-phase progression of infected cells was inhibited by AAV Rep78 via hypophosphorylation of Rb [90]. In addition, the AAV2 genome was shown to trigger DNA damage, signaling that resembles the response to an aberrant cellular DNA replication fork [91]. The formation of DNA damage-response foci induced by the AAV genome strictly depends on ATM and Rad3-related protein (ATR), Chk1 and DNA topoisomerase 2-binding protein 1, but not ATM or NBS1 [91]. The p5 promoter sequence of AAV2 was identified to be responsible for inducing the host DNA damage response, which leads to G2/M arrest [92]. Furthermore, in p53-deficient cells, UV-inactivated AAV2 triggered mitotic catastrophe associated with a dramatic Chk1-dependent

overduplication of centrioles, and the consequent formation of multiple spindle poles in mitosis [93]. In p53-proficient cells, H2AX was indispensible for the formation and maintenance of DNA repair foci induced by stalled replication and p21-mediated cell cycle arrest [94]. Moreover, the activation of H2AX was shown to be the result of ATR overactivation and diffusion, but independent of ATM.

Preferentially, AAV induces apoptotic cell death in p53-deficient cells, which lack the ability to maintain prolonged G2/M arrest via Chk1-mediated mitotic catastrophe. In p53-proficient cells, AAV infection-induced G2/M arrest was maintained via the ATR–Chk1–H2AX–p53–p21 pathway, without resulting in significant cell death. During coinfection with adenovirus, however, replication of the AAV2 genome induced a DNA damage response through the activation of the primary mediator, DNA protein kinase C [95,96].

Parvovirus

Cell death induced by members of the genus *Parvovirus* is cell-type dependent. In tissue samples of cats and dogs with panleukopenia and enteritis, respectively, apoptosis has been shown to contribute significantly to the widespread tissue damage caused by parvovirus infection [97]. Apoptotic cell death induced by H-1 parvovirus (H-1PV) has been reported [98–100]; however, necrosis also has been shown to mediate the H-1PV-induced CPE in apoptosis-resistant cell types [101], and murine minute virus (MVM) induced CPE in permissive murine A9 cells [102].

The oncolytic potential of rodent parvoviruses, particularly MVM and H-1PV, has been drawing attention for decades. A rich body of studies has emphasized the CPE induced by MVM and H-1PV, which is selectively seen in tumorized cells, but not their normal counterparts. The selective killing of tumorized cells by MVM and H-1PV was shown *in vitro* in cultured SV40-transformed cells over 20 years ago [103,104]. A series of animal experiments showed the suppressive effect of MVM and H-1PV on different tumors or tumor grafts [2]. Stimulated parvovirus lifecycle, or lytic activation in tumorized cells versus normal counterparts, was proposed as the mechanism behind the oncolytic function, based on the observation that a higher level of viral replication and production was achieved in tumorized cells than in nontransformed cells [105,106]. This hypothesis is sound, given that S-phase, which is pivotal for parvovirus replication, is more active in tumorized cells. On the other hand, the NS1 protein expression level and phosphorylation status, which differs in tumorized cells versus normal counterparts, may also contribute to the selective killing function [107–110].

The CPE induced by MVM infection, shown mainly to affect micro and intermediate filaments of the cytoskeleton network, while the nuclear lamina and microtubules remain intact throughout infection [111], is mainly caused by the activity of NS1 [112]. The interaction of MVM NS1 and casein kinase II (CKII) was revealed to mediate CKII-dependent cytoskeletal alterations and nonapoptotic cell death in murine A9 fibroblasts [113]. NS1 acted as an adaptor molecule, linking the cellular protein kinase CKIIα to tropomyosin and, thus, modulating the substrate specificity of the kinase. This action results in an altered tropomyosin phosphorylation pattern, both *in vitro* and in living cells. Moreover, NS1 mutants that abolished binding with either CKIIα or tropomyosin lost the capacity to induce CPE. The fusion peptides, in which the tropomyosin-binding domain of NS1 and CKIIα are physically linked, were able to mimic NS1 in its ability to induce the death of transformed MVM-permissive cells. In addition, the Raf-1 signaling control of nuclear transport was suggested to be the target during MVM infection-induced oncolysis [114].

As for H-1PV, necrosis was detected in infected glioma cells, which are resistant to cisplatin and TNF- related apoptosis-inducing ligand (TRAIL)-induced apoptosis. H-1PV has been found to kill glioma cells via a nonapoptotic cell death mechanism, mediated by cathepsins [101]. Lysosomal membrane permeabilization, and the resulting release of lysosomal enzymes, and, in particular, cathepsins, into the cytosol, is the main pathway to mediate this type of cell death [115,116]. By contrast, in a recent report, H-1PV NS1 was shown to induce apoptotic cell death via the induction of reactive oxygen species in 293 cells, which is inconsistent with the observation in H-1PV infection of NB324K, a SV40-transformed human cell line [117]. Inhibition of reactive oxygen species by a different reducing compound significantly decreased NS1-induced apoptosis. More importantly, H-1PV kills human hepatocellular carcinoma cell line (QGY-7703) cells by a non-apoptotic process; however, a cDNA microarray analysis of H-1PV-infected cells showed that genes involved in signal transduction, apoptosis, DNA replication, DNA repair, DNA binding and transcription were differentially expressed after H-1PV infection [118].

As seen in other genera, members of the genus *Parvovirus* are also able to induce cell cycle arrest during infection. An increased S/G2/M-phase population was observed during MVM infection [119–122]. G2/M arrest was also detected during H-1PV infection [117]. MVM-induced cell cycle arrest in S-phase was shown to be p53-dependent, but $p21^{cip1}$-independent, whereas the arrest in G2 was dependent on both p53 and its downstream effector $p21^{cip1}$ [122]. The MVM NS1 protein alone was able to reproduce cell cycle arrest, as seen during MVM infection [120,122]. An early study implied one of the possible mechanisms underlying MVM NS1-induced cell cycle arrest was the direct nicking of the host cell chromatin by NS1, which took place hours before the cell cycle arrest [121]. The damaged chromatin DNA might induce a DNA damage response, which, in turn, stalls the cell cycle for repair. Indeed, a DNA damage response, together with cell cycle arrest and apoptosis, was detected during H-1PV infection [117], as well as during MVM infection [Pintel D, Pers. Comm.]. However, whether the viral genome or the nicked host chromatin triggered the DNA damage response remains unanswered.

Taken together, the CPE induced by members in the genus *Parvovirus* could be either necrosis or apoptosis, depending on the virus and cell type. The NS1 protein clearly plays a role in inducing cell death and the cell cycle arrest of infected cells via multiple strategies.

Discussion

Parvoviruses induce both cell death and cell cycle arrest during infection. While necrosis was reported during BPV, MVM and H-1PV infection, apoptosis has been demonstrated as the major pathway, mediating parvovirus infection-induced cell death. The cell cycle perturbation during parvovirus infection mainly appears as G2/M-phase arrest. However, S-phase plateau was also reported during MVC infection through an accumulated/prolonged S-phase [74].

From an immunological standpoint, apoptosis induced during virus infection is thought to be a means for the host to defend itself against viral invasion [123]. However, apoptosis may also represent a crucial step in the viral cell lifecycle and pathogenesis [124,125], as apoptotic cell death allows for viral release and induces less immune response than cell lysis, which releases cellular components and triggers an inflammation response [126]. For parvovirus, caspase activation is clearly required to regulate DNA replication and virion production through caspase-mediated specific cleavage of AMDV NS1 and VP2 [19,68]. Apoptotic cell death apparently is a direct cause of diseases during parvovirus infection, especially anemia and fetal death induced during B19V infection [29,30] and, possibly, gastroenteritis and pneumonia caused by parvovirus [27]. The oncolytic effect of AAV relies

on the selective induction of apoptosis in p53-deficient or tumorized cells [127]. It may not be critical for the virus lifecycle in natural coinfection with adenovirus [95]; however, its potential implication is valuable. MVM and H-1PV can induce cell death in a number of tumor cells while having no cytotoxic effect on healthy tissues. The mechanism underlying specific cell death of tumorized cells by these viruses is still unclear. H-1PV infection can induce either necrosis or apoptosis, depending on the types of tumorized cells [99,128,129].

Virus infection-induced cell cycle arrest has been extensively documented, both in DNA and RNA viruses [130,131]. The S-phase is generally critical for parvovirus replication, which takes place in the nucleus and utilizes host polymerase [132–135]. H-1PV protein synthesis coincides with the cellular DNA synthesis [136], while MVM replication has been shown to require mitotically active cells [137]. B19V showed a maximal rate of transcription preceding the onset of S-phase-dependent replication of the viral genome [138]. The prevention of S-phase was reported to decrease the gene targeting of AAV2 [139]. Although AAV2 infection has not been reported to elicit S-phase arrest [91,140,141], the AAV2 Rep78 was shown to inhibit Cdc25A and arrest the transfected cells at S-phase [142]. Thus, it is reasonable to speculate that parvoviruses have the common feature of prolonging the S- or G2-phase in favor of their replication. The G2/M-phase arrest is more likely to be a pseudo S-phase, with stalled host DNA replication but an active environment to facilitate viral DNA replication [130]. The postmitotic arrest seen during AMDV infection indicates that the active DNA replication environment is maintained at cell cycle checkpoint arrest [70]. In concert, active 5-bromo-2-deoxyuridine incorporation was seen in parvovirus-infected cells with apparent G2/M-phase arrest [90,121].

Cell cycle disturbance and programmed cell death are two relatively separate events; however, increasing evidence suggests their interactive crosstalk. Prolonged cell cycle arrest can induce apoptosis [143,144], which is mediated through cyclin/CDK and Bcl2 family proteins [145]. A balance between cell cycle checkpoint maintenance and apoptosis is finely regulated during DNA damage to ensure the accuracy of replication and minimize the unwanted loss [146]. The terminal repeat structures of parvoviruses can be easily detected as damaged DNA, thus triggering a DNA damage response, which, in turn, induces cell cycle arrest for ‘repair’. As replication of parvovirus requires cellular DNA polymerase [147], parvovirus replication favors cells in S-phase, in which active polymerase and other replication factors are present [148], it is logical to hypothesize that parvoviruses use parts of the single-stranded genome, especially the terminal repeats and/or the left-hand promoter region, as triggers to induce a DNA damage response to prolong the S- and G2-phase during the early stage of infection, but not induce apoptosis. At this stage, active DNA polymerase and other necessary replication factors are present, and these are used for genome replication. This hypothesis is supported by the fact that inhibition of DNA damage response significantly reduced AAV Rep expression [95], as well as MVC [Luo Y, Qiu J, Unpublished Data] and MVM replication [Pintel D, Pers. Comm.]. At the late stage of infection, the collaboration of ‘irreparable damage’ and viral proapoptotic protein breaks the balance created by checkpoint arrest and leads to apoptosis, which is beneficial for viral egress. In addition, apoptosis is also probably contributed from a cellular mechanism to defend virus infection if the virus infection cannot be cleaned or limited in the cells.

Conclusion

The CPE induced during parvovirus infection is mediated either by apoptosis or necrosis, and is contributed by cell cycle arrest, mostly at G2/M. While cell cycle arrest and caspase activation create an optimum environment for parvovirus replication at the early stage of infection, the ultimate apoptosis is believed to be beneficial for virus egress at the late stage. The mechanism underlying induction of cell cycle arrest is probably owing to a DNA

damage response induced by the viral genome via the terminal repeats and left-hand promoter region. Yet, apoptosis could be the ultimate consequence of the proapoptotic activity of viral proteins, prolonged cell cycle arrest and irreparable DNA damage response to ensure the optimum release of virion. Interestingly, parvovirus infection also uses a novel necrotic mechanism to induce cell death when apoptotic cell death cannot be achieved during virus infection, as seen in H-1PV infection, where the cell death mechanism varies depending on the host. Thus, parvovirus infection may provide a unique model to investigate the virus–host interaction network, which fine tunes the balance among viral DNA replication, DNA damage response, cell cycle arrest and cell death.

Future perspective

B19V is the only parvovirus that has been confirmed to be pathogenic to humans. The study of host–virus interaction has long been hampered by the lack of a permissive culture system. With the establishment of the highly permissive $CD36^+$ erythroid progenitor cells, a new wave of B19V research is around the corner.

Several new human-hosted members of *Parvovirinae* have been identified recently (e.g., HBoVs and human parvovirus 4). However, the disease association of these viruses is currently under active investigation. The biology and pathogenesis of these viruses awaits further exploration.

The oncolytic property of the rodent parvoviruses (i.e., MVM and H-1PV) has been extensively explored for their potential application in human cancer therapy. A step toward this goal is to assess the toxicity of these viruses to the host (e.g., the immune system). In order to apply the oncolytic parvoviruses to the clinic, they must be further optimized to be systemically nontoxic.

Parvoviruses are among the smallest viruses in the nature. Parvovirus infection usually induces programmed cell death and cell cycle arrest. DNA damage response during viral invasion has been increasingly reported. Taking advantage of their short genome and relatively simple gene-expression profile, the parvovirus infection system may provide a unique tool to study the crosstalk between cellular mechanisms (e.g., cell death, cell cycle regulation and DNA damage response).

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▪▪ of considerable interest

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