The small 11kDa non-structural protein of human parvovirus B19 plays a key role in inducing apoptosis during B19 virus infection of primary erythroid progenitor cells

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Abstract

Human parvovirus B19 (B19V) infection shows a strong erythroid tropism and drastically destroys erythroid progenitor cells, thus leads to most of the disease outcomes associated with B19V infection. In this study, we systematically examined the three B19V nonstructural proteins, 7.5kDa, 11kDa and NS1, for their function in inducing apoptosis in transfection of primary ex vivo expanded erythroid progenitor cells, in comparison with apoptosis induced during B19V infection. Our results show that 11kDa is a more significant inducer of apoptosis than NS1, while 7.5kDa does not induce apoptosis. Furthermore, we determined that caspase-10, an initiator caspase in death receptor signaling, is the most active caspase in apoptotic erythroid progenitors induced by 11kDa and NS1 as well as during B19V infection. More importantly, cytoplasm-localized 11kDa is expressed at least 100 times more than nucleus-localized NS1 at the protein level in primary erythroid progenitor cells infected with B19V; and inhibition of 11kDa expression using anti-sense oligos targeting specifically to the 11kDa-encoding mRNAs reduces apoptosis significantly during B19V infection of erythroid progenitor cells. Taken together, these results demonstrate that the 11kDa protein contributes to erythroid progenitor cell death during B19V infection.
Introduction

B19V infection is the cause of "fifth disease", a highly contagious infection of childhood. B19V infection can result in serious and occasionally fatal hematologic diseases in susceptible patients.\textsuperscript{1} In patients with increased destruction of red cells and a high demand for the production of erythrocytes, acute B19V infection can cause transient aplastic crisis. Pure red-cell aplasia can also be a manifestation of persistent B19V infection in immunocompromised or immunodeficient patients.

B19V belongs to the genus \textit{Erythrovirus} in the family \textit{Parvoviridae}.\textsuperscript{2} Spanned by two identical terminal hairpin repeats, the 5.6-kb linear single stranded DNA genome of B19V encodes a single nonstructural protein (NS1), and two capsid proteins (VP1 and VP2). Two other smaller non-structural proteins, 7.5kDa and 11kDa, have been detected during B19V infection.\textsuperscript{3,4} The 11kDa protein is translated from a small left-ORF that overlaps with the C-terminal of the VP1/VP2 ORF in a different frame. The 7.5kDa protein is translated from a small mid-ORF. NS1 is a multiple functional polypeptide essential to viral replication and regulation of gene expression that is cytotoxic to host cells.\textsuperscript{5-8} The 11kDa protein has been shown to have a role in virion production and trafficking in infected cells, while the 7.5kDa protein has not yet been reported to have functions during B19V infection.\textsuperscript{9}

B19V shows extreme tropism for erythroid progenitor colony forming unit-erythroid cells (CFU-E) and burst-forming unit-erythroid cells (BFU-E) in the bone marrow of patients.\textsuperscript{10-12} Disease manifestations of B19V infection, as seen in transient aplastic crisis, pure red cell aplasia and hydrops fetalis, are due to the direct cytotoxicity of the virus,\textsuperscript{13} a direct outcome of the cell death of erythroid progenitors that are targets of B19V replication. A progressive host cell apoptosis has been identified during B19V infection of primary erythroid progenitor cells and megakaryoblastoid cell lines.\textsuperscript{5,14} NS1 expression in megakaryoblastoid cell lines has been associated with B19V-induced apoptosis;\textsuperscript{5,14} however, the kinetics of NS1 expression has not correlated with that of induced apoptosis during B19V infection of megakaryoblastoid cell line
UT7/Epo-S1, which are semi-permissive to B19V infection. These findings raise the question about the role of NS1 in inducing apoptosis during B19V infection.

In the present study, we show for the first time that 11kDa is a more significant inducer of apoptotic cell death than NS1 in transfection of primary erythroid progenitor cells. Since 11kDa is expressed at least 100 times more than NS1 at the steady-state protein level in erythroid progenitor cells during B19V infection, we conclude that the B19V infection-induced apoptosis of erythroid progenitor cells, is largely mediated by the small non-structural 11kDa protein.

**Materials and Methods**

**Cells and virus infection:**

HeLa cells, 293 cells, K562 cells and UT7/Epo-S1 cells were maintained as previously described. Human primary CD36+ erythroid progenitor cells (CD36+ EPCs) were expanded ex vivo in the expansion medium as previously described. Large numbers of CD36+ EPCs, which were used for either transfection or B19V infection, were obtained on day 8 or day 9. The animal protocol for producing antibodies was approved by the IACUC at the University of Kansas Medical Center.

Twenty microliters of B19V viremic plasma that contained $1 \times 10^{12}$ copies of B19V genome per ml was incubated with $2 \times 10^6$ cells, in a volume of 500 µl medium with slow rotation at 4°C for 2 hrs. Infected cells were then cultured in the expansion medium at a concentration of $2 \times 10^5$ cells/ml at 37°C with 5% CO$_2$. 
Caspase inhibitors:

Two general caspase inhibitors, pan-caspase fmk Inhibitor Z-VAD-fmk (Z-VAD) and Oph inhibitor Q-VD-Oph (Q-VD), and nine individual caspase inhibitors (caspase-1, -2, -3&7, -4, -6, -8, -9, -10 & -13 inhibitors) were purchased from R&D Systems (Minneapolis, MN).

Morpholino oligos:

Three Morpholino anti-sense oligos were designed to specifically target sequences in the region of the AUG translation start site of the 11kDa-encoding mRNAs, which are diagramed in Fig. 5A. Their sequences written from 5’ to 3’ and complementary to the 11kDa-encoding mRNA are as follows: MO-1: TCTTCAGGCTTTTCATATCCATGTC; MO-2: CCATGTCTGTGGTGTTTGCAT and MO-3: TGTAGGTTGCTACGAACTGGTCTGC. The Morpholino oligos were synthesized at Gene Tools, LLC (Philomath, OR), and Endo-Porter was used for delivery following a manufacturer protocol. A random control Morpholino was used as a control.

Plasmid construction:

NS1, 7.5kDa and 11kDa expression plasmids in mammalian cells: GFP was cloned into pNTAP-B (Strategene) by BamH I and EcoR I as pGFP. Then we cloned the NS1 ORF (nt 616-2631), the 7.5kDa mid-ORF (nt 2090-2305) and the 11kDa ORF (nt 4890-5171) into this pGFP plasmid through EcoR I/Xho I sites as pGFP-NS1, pGFP-7.5kDa and pGFP-11kDa, respectively. pRFPHA, pRFP-NS1HA and pRFP-11kDaHA were constructed by replacing GFP with RFPHA (C-terminal HA tagged red fluorescent protein, DsRed, Clontech), RFP-NS1HA and RFP-11kDaHA (NS1 and 11kDa were HA tagged at C-terminal) in the pGFP, respectively.
Bacterial expression plasmids of glutathione S-transferase (GST) fused B19V proteins: The 11kDa ORF and the N-terminus encoding sequence (nts 616-1158) of the NS1 were cloned into pGEX4T3 (GE Health) as pGEX11kDa and pGEXNS1(aa1-181), respectively. All the nucleotide (nt) numbers refer to the sequence of the B19V J35 isolate (Genbank accession no.: AY386330).

Reverse transcription (RT)–real time PCR: A multiplex RT-real time PCR system was performed to detect B19V 11kDa-encoding and NS1-encoding mRNAs, with β-actin mRNA serving as an internal control as previously reported.19,20

Production of antisera against B19V non-structural proteins: GST- fused full length 11kDa (GST-11kDa) and NS1 amino acids (aa) 1-181 [GST-NS1(aa1-181)] were expressed and purified as we previously described.21 Polyclonal production was performed following protocols as previously described.21

Transfection: The 293 and Hela cells were transfected with 2 µg of DNA per 60-mm dish using Lipofectamine and Plus reagent (Invitrogen) as previously described.22 K562 cells were electroporated with 2 µg of DNA per 2 × 10^6 cells using reagent V and program T6 with the Amaxa® Nucleofector® (Lonza Inc.). UT7/Epo-S1 cells and CD36+ EPCs were electroporated with 2 µg of DNA per 2 × 10^6 cells, using a universal transfection reagent with program X-005 as
previously described. After transfection, CD36+ EPCs were maintained in the expansion medium.

**SDS-PAGE, Western blotting and immunofluorescence:**

SDS-PAGE, Western blotting and immunofluorescence assay were performed as previously described.

**Flow cytometry analysis:**

**AnnexinV/Propidium Iodide (PI) staining:** Cells were stained alive with Cy5-conjugated AnnexinV (BD Biosciences) and PI (Sigma) together to detect apoptotic cells according to the manufacturer’s instructions (BD Biosciences).

**TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling) assay:**

TUNEL assay was basically performed according to the manufacturer’s protocol (MBL International, MA) with the modification that streptavidin-Cy5 (Jackson ImmunoResearch, PA) was used to develop fluorescence.

**FLICA (Fluorochrome Inhibitor of Caspase Assay):** Live cells (1 × 10^6) were stained with respective FAM-labeled FLICA peptide according to the manufacturer’s manual (Immunochemistry Tech, MN).

All the samples were analyzed on the three-laser flow cytometer (LSR II, BD Biosciences) within an hour of staining at the Flow Cytometry Core on campus. All flow cytometry data were analyzed using FACS DIVA software (BD Biosciences).

**Results**

**11kDa induces apoptosis in both B19V permissive and nonpermissive cells.**

To examine the potential role of 11kDa in inducing apoptosis, we transfected pGFP-11kDa and pGFP plasmids, respectively, into both B19V permissive and nonpermissive cells.
The GFP positive (GFP+) population was selectively gated and analyzed in comparison with the GFP negative [GFP(-)] population using AnnexinV/PI double staining. There were significantly more AnnexinV positive (AnnexinV+) cells in GFP-11kDa expressing UT7/Epo-S1, CD36+ EPCs, HeLa and K562 cells than these in GFP control expressing cells (Fig. 1A&B, GFP+), but no significant difference was observed between the GFP-11kDa and GFP expressing 293 cells (Fig. 1A&B, GFP+ 293).

In UT7/Epo-S1 cells, the population of AnnexinV+ cells induced by GFP-11kDa reached a high rate of 48.0% at 48 hrs posttransfection in GFP+ cells (Fig. 1A, GFP+ S1). In contrast, GFP(-) cells in the same sample showed only 5.1% AnnexinV+ cells [Fig. 1A, GFP(-) S1]. Less than 13.1% of GFP+ cells in the pGFP-transfected cells (as control) were stained with AnnexinV (Fig. 1B, GFP+ S1). GFP-11kDa induced an average of 37.1% more AnnexinV+ cells in GFP+ cells than in GFP(-) cells at 48 hrs posttransfection (Fig. 1C, GFP-11kDa S1), indicating that the AnnexinV+ population of GFP+ cells in the pGFP-11kDa-transfected cells is predominately induced by the 11kDa, not the GFP.

The GFP-11kDa also induced a high AnnexinV+ population in CD36+ EPCs that are highly permissive to B19V infection in vitro and are the primary cells most closely resembling the CFU-E and BFU-E in the bone marrow of patients. Similar to UT7/Epo-S1 cells, GFP-11kDa induced an average of 42.6% more AnnexinV+ cells in GFP+ cells than in GFP(-) cells at 24 hrs posttransfection (Fig. 1C, GFP-11kDa CD36+). In comparison, pGFP control transfection only resulted in approximately 7% AnnexinV+ cells over the background (Fig. 1C, GFP CD36+). Notably, we observed a significant amount of background cell death in transfected CD36+ EPCs, which presumably was induced by the electroporation and despite the optimized conditions that allowed us to transfec the CD36+ EPCs. At 24 hrs posttransfection, as much as 34% of the background AnnexinV+ population was detected in GFP(-) cells of pGFP-transfected CD36+ EPCs [Fig. 1B, GFP(-) CD36+]. This amount increased to more than 50% at 48 hrs posttransfection, which made assessing apoptosis induced by transfection of the GFP-11kDa
less accurate (data not shown). Therefore, we chose to assay transfected CD36+ EPCs only at 24 hrs posttransfection.

The 11kDa also induced a significant amount of AnnexinV+ cells in B19V nonpermissive cells, HeLa and K562 cells, in addition to these B19V permissive cells. We observed more than 30% of AnnexinV+ cells in GFP+ population of the pGFP-11kDa-transfected cells of both types at 48 hrs posttransfection (Fig. 1A, GFP+ HeLa&K562). Conversely, pGFP transfection alone only induced approximately 9% and 6% of the AnnexinV+ population in GFP+ HeLa and K562 cells, respectively (Fig. 1B, GFP+ HeLa&K562). However, the GFP-11kDa was not able to induce a significant amount of AnnexinV+ population in 293 cells (Fig. 1 A&C, 293); E1b-19kDa protein that is expressed in 293 cells perhaps inhibits apoptosis in a way similar to that of Bcl-xL.25,26 In the pGFP transfection control of all the five cell types, GFP did not significantly induce the AnnexinV+ population in GFP+ cells of transfected cells in comparison with that in GFP(-) cells (Fig. 1B). When we transfected the CD36+ EPCs, however, we noted a relatively high percentage of AnnexinV+ population in GFP(-) cells of both pGFP-11kDa- and pGFP-transfected cells (Fig. 1A&B, CD36+), which indicated background cell death that was caused in part by the electroporation.

The majority of AnnexinV+ cells induced by GFP-11kDa were PI stained negative [AnnexinV+/PI(-)], and thus were in the early or middle stage of apoptosis. Specifically, approximately 80% of AnnexinV+ cells were PI stained negative in UT7/Epo-S1, HeLa and K562 cells, and 60% were PI stained negative in CD36+ EPCs [Fig. 1C, AnnexinV+/PI(-)]. The cell population of AnnexinV+/PI+ is an indicator of either late apoptosis or necrosis (Fig. 1C, AnnexinV+/PI+). In addition, we directly visualized a representative cell with GFP-11kDa expression with distinct cellular nucleus degradation. We also observed small apoptotic bodies enclosing a degraded nucleus by green fluorescence (Fig. 1D).27,28 Overall, these results suggest that the 11kDa induces cell death with apoptotic features rather than necrosis in both
B19V permissive and nonpermissive transfected cells. Further supporting this finding, 11kDa did not induce an AnnexinV+ cell population in 293 cells.

11kDa is a more significant inducer of apoptosis than NS1.

The apoptotic nature of NS1 has not been examined in primary erythroid progenitor cells. We sought to determine which protein, 11kDa or NS1, was more potent in inducing apoptosis in UT7/Epo-S1 cells and CD36+ EPCs. For comparison, we also studied the 7.5kDa, another small nonstructural protein shown to be expressed during B19V infection but with an unknown function.3

While the GFP-11kDa and the GFP-NS1 both extensively induced AnnexinV+ cells when transfected to UT7/Epo-S1 cells, the GFP-7.5kDa poorly induced the AnnexinV+ population in GFP+ cells (Fig. 2A). In a time-dependent manner, the GFP-11kDa caused the AnnexinV+ population to increase from less than 20% to more than 60%, at 24 to 72 hrs posttransfection (Fig. 2A). At all three time points, the extent of the GFP-11kDa-induced AnnexinV+ population was significantly higher than that induced by the GFP-NS1 (Fig. 2A). However, at 72 hrs posttransfection only, an increased population of AnnexinV+ cells, approximately 16%, was observed in pGFP-7.5kDa-transfected cells compared with the pGFP-transfected control (8%) (Fig. 2A). To better evaluate the potency of the NS1 and the 11kDa in inducing apoptosis, we normalized the level of AnnexinV+ population by the level of protein expression represented by the mean intensity of the green fluorescence of the GFP detected by flow cytometer (Fig. 2B).29-31 Results normalized by GFP were plotted as relative values to that of the GFP+ population of pGFP-11kDa-transfected cells, which was arbitrarily set to 100% (Fig. 2C). Transfecting the GFP-11kDa induced approximately two-fold more AnnexinV+ cells than transfecting the GFP-NS1 did (Fig. 2C), indicating that the 11kDa is twice more potent in inducing apoptosis than the NS1 in UT7/Epo-S1 cells. However, the difference between the GFP control and GFP-7.5kDa
transfection groups was not statistically significant after normalization, suggesting that the 7.5kDa is not a significant apoptosis inducer in UT7/Epo-S1 cells.

Similar results were obtained by transfecting CD36+ EPCs (Fig. 2D). At 24 hrs posttransfection, while pGFP control transfection induced approximately 10% AnnexinV+ cells over the background, transfection of pGFP-7.5kDa, pGFP-NS1 and pGFP-11kDa induced a significantly higher level of the AnnexinV+ population, approximately 22%, 36% and 42%, respectively (Fig. 2D). After normalized by the GFP expression level (Fig. 2E), the GFP-11kDa induced approximately 1.5 times more AnnexinV+ cells than did the GFP-NS1 (Fig. 2F), results similar to those obtained with UT7/Epo-S1 cells (Fig. 2C). Again, the difference between the GFP-7.5kDa and GFP alone did not continue to be statistically significant after normalization (Fig. 2F). Given the unique nature of ex vivo culture of CD36+ EPCs (even the GFP control induced about 10% AnnexinV+ cells over the background), we determined that the GFP-7.5kDa was not a significant inducer of apoptosis, and thus focused our study on the NS1 and the 11kDa thereafter.

For comparison, we also determined the apoptosis level represented by TUNEL positive (TUNEL+) population during B19V infection of CD36+ EPCs. B19V infected positive (capsid+) population was specifically selected to assess the extent of nicked-DNA-containing cells, compared to those of the capsid negative [capsid(-)] population. As shown in Fig. 2G, at 24, 48 and 72 hrs pi, about 20%, 50% and 64% TUNEL+ populations were detected, respectively, in the capsid+ population. Interestingly, we also found a time-dependent increase of TUNEL+ population in the B19V uninfected infected [capsid(-)] cell population, from 2% at 24 hrs p.i. to 19% at 48 hrs p.i., which rose to more than 25% at 72 hrs p.i. The TUNEL+ populations in capsid(-) cells is likely due to the sensitivity of the capsid-recognizing antibody, but not to the release of apoptosis-inducing molecules from infected cells (Fig. S4). However, a significant difference was consistently found between the capsid+ and capsid(-) cell populations. Similar results were obtained when the NS1-expressed cell population was selected for TUNEL assay.
using the anti-NS1 sera (data not shown). Thus, our results confirmed the apoptotic nature of CD36+ EPCs during B19V infection.

**11kDa localizes dominantly in cytoplasm and is expressed at least 100 times more than NS1 during B19V infection of CD36+ EPCs.**

Induction of apoptosis is often caused by accumulation of the apoptotic inducer in the cytoplasm, and nuclear translocation is often a means to inactivate the apoptotic inducer.\(^{32,33}\) By using anti-NS1 (αNS1) and anti-11kDa (α11kDa) specific sera (Fig. 3A), GFP-11kDa and GFP-NS1 in transfected UT7/Epo-S1 cells and CD36+ EPCs showed similar cellular localization as the 11kDa and the NS1 expressed in B19V-infected CD36+ EPCs (Fig. 3B&C). The blue nuclear DAPI staining did not overlap with either the green GFP-11kDa (Fig. 3B) or the 11kDa stained with α11kDa (red) (Fig. 3C), indicating that the GFP-11kDa and the 11kDa localize predominantly in cytoplasm. Conversely, nuclear DAPI staining overlapped exactly with NS1 stained with αNS1 (red) in B19V-infected CD36+ EPCs (Fig. 3C), confirming that NS1 is expressed exclusively in nucleus in B19V infected cells as previously reported.\(^{9,34}\) In pGFP-NS1-transfected UT7/Epo-S1 cells and CD36+ EPCs, the GFP signal diffused to cytoplasm to some extent, however, the GFP-NS1 localized mainly in the nucleus.

We next compared the expression level of GFP-11kDa and GFP-NS1 with that of 11kDa and NS1, respectively, during B19V infection. The level of the GFP-11kDa in transfected UT7/Epo-S1 cells and CD36+ EPCs at 48 hrs posttransfection, as quantified by flow cytometry analysis using α11kDa antiserum, was approximately 12 times lower than that of the 11kDa expressed in B19V-infected CD36+ EPCs at 48 hrs p.i. (Fig. 3D, α11kDa), implying that a stronger proapoptotic effect is induced by 11kDa in B19V-infected CD36+ EPCs than that induced by the GFP-11kDa in transfected cells. In contrast, nearly twice as much GFP-NS1 was expressed in both transfected UT7/Epo-S1 cells and CD36+ EPCs cells at 48 hrs posttransfection than the NS1 expressed in B19V-infected CD36+ EPCs at 48 hrs p.i. (Fig. 3D,
αNS1), indicating that the GFP-NS1 in transfected cells likely mimics the function of the NS1 during B19V infection.

To determine the relative expression level of 11kDa and NS1 during B19V infection of the native targets, erythroid progenitor cells, we quantified the mRNA levels of the two non-structural proteins using RT-real time PCR. By normalizing to copy numbers of β-actin mRNA (relative copies per β-actin mRNA), the 11kDa-encoding mRNA remained at a consistent level that was approximately 100 to 200 times higher than that of the NS1-encoding mRNA during the course of B19V infection (Fig. 4A).

Further, to ascertain the steady-state protein level of 11kDa and NS1 during B19V infection, we attempted to estimate the relative protein level of 11kDa vs. NS1 during B19V infection. GST-NS1(aa1-181) and GST-11kDa were purified (Fig. 4B). The purified protein standards (Fig, 4C, lanes 1-5 and Fig. 4D, lanes 1&2, respectively) and cell lysates from B19V-infected CD36+ EPCs (at 48 hrs p.i.) and mock cells were blotted with α11kDa and αNS1 antisera, respectively (Fig. 4C&D). We observed significant nonspecific protein bands by the αNS1 antiserum; however, the blots clearly showed the specific NS1 band with both 8% and 6% PAGE gels (Fig. 4C, compare lane 6 with 7 and lane 8 with 9, respectively). The intensity of this specific NS1 band from B19V-infected CD36+ EPCs (Fig. 4C, lane 6) fell between 1.0 ng and 0.33 ng of the GST-NS1(aa1-181) standards (Fig. 4C, lanes 4&5). In contrast, the signal of the 11kDa from B19V-infected CD36+ EPCs was stronger than that from 100 ng of the GST-11kDa (Fig. 4D, compare lane 3 with 1). This result suggests that during B19V infection of CD36+ EPCs at 48 hrs p.i., at a steady-state protein level, 11kDa expresses at least 100 times more than NS1 (Fig. 4C&D), which presented during the course of B19V infection of CD36+ EPCs (Fig. S2).

Both high expression and cytoplasmic localization of 11kDa and the low expression and nuclear localization of NS1 during B19V infection of CD36+ EPCs suggest the important role of the 11kDa in apoptosis of B19V-infected erythroid progenitors.
Inhibition of 11kDa expression by 11kDa-specific Morpholinos reduces apoptosis significantly during B19V infection of CD36+ EPCs.

To confirm a key role of 11kDa in inducing apoptosis during B19V infection, we next applied specific Morpholino anti-sense oligos to knock down 11kDa expression through inhibition of translation initiation. CD36+ EPCs were pre-treated with Morpholino oligos 24 hrs prior to infection. The expression of 11kDa was reduced by approximately 60% in B19V-infected CD36+ EPCs treated with MO-1, MO-2 and MO-3 at 48 hrs p.i., as indicated by Western-blot (Fig. 5B). Consequently, as a result, the level of apoptosis, indicated by TUNEL+ population, was reduced by approximately 20%, in comparison with the cells treated with the control Morpholino (MO-Ctrl) (Fig. 5C). The expression levels of NS1 and capsid protein VP1 and VP2 were not affected with treatment of Morpholinos as determined (data not shown), indicating MO-1, MO-2 and MO-3 target specifically to the 11kDa-encoding mRNAs. Thus, we have demonstrated that inhibition of 11kDa expression reduces apoptosis during B19V infection of CD36+ EPCs, supporting a key role of the 11kDa in inducing apoptosis during B19V infection.

Caspase-10 inhibitor is as effective as pan-caspase inhibitors in reducing B19V-induced apoptosis.

Transfecting UT7/Epo-S1 cells with GFP-11kDa, we observed an 80% inhibition of AnnexinV+ population at 48 hrs posttransfection when Q-VD, a newly developed pan-caspase inhibitor without cross-inhibition of cathepsin, was used at 10 µM (Fig. 6A). Among individual caspase inhibitors (caspase-1, -2, -3&7, -4, -6, -8, -9, -10 and -13 inhibitors), the caspase-10 inhibitor was particularly effective; treatment with caspase-10 inhibitor at 20 µM reduced the percentage of the AnnexinV+ population by 55%. However, treatments with caspase-1, -2, -3&7, -4, -6 and -8 inhibitors at 20 µM only reduced the AnnexinV+ population by approximately 20%. The inhibition of the GFP-NS1-induced AnnexinV+ population in UT7/Epo-S1 cells generally
shared the same profile but had less sensitivity compared with the population induced by 11kDa. At 48 hrs posttransfection, less inhibition was observed in pGFP-NS1-transfected cells with the treatment of the same dose of inhibitors; treatments with Q-VD, Z-VAD and caspase-10 inhibitor reduced the AnnexinV+ population by 55%, 45% and 40%, respectively (Fig. 6A). Notably, after transfecting both UT7/Epo-S1 cells and CD36+ EPCs, the percentage of the GFP+ population (out of the total) in pGFP-NS1-transfected cells was approximately 1.6 times more than that of pGFP-11kDa-transfected cells as determined by flow cytometer (data no shown). This perhaps can partially explain why pGFP-NS1-transfected cells were less sensitive to these caspase inhibitors. Nevertheless, caspase-10 inhibitor clearly was the most effective in inhibiting both GFP-11kDa- and GFP-NS1-induced apoptosis in transfected UT7/Epo-S1 cells.

As expected, a similar inhibitory effect of all the caspase inhibitors was observed after transfecting GFP-11kDa and GFP-NS1 in CD36+ EPCs (Fig. 6B). Q-VD treatment showed the strongest inhibition in both the GFP-11kDa- and the GFP-NS1-induced AnnexinV+ cells, followed by Z-VAD and caspase-10 inhibitor. Similar to UT7/Epo-S1 cells, the GFP-NS1-induced AnnexinV+ cells were less sensitive to inhibition caused by caspase inhibitors. For example, caspase-10 inhibitor reduced the GFP-11kDa-induced AnnexinV+ population by 62%; however, the NS1-induced AnnexinV+ population was only inhibited by 45%.

In B19V infection of CD36+ EPCs, Q-VD was also the most effective inhibitor in decreasing the TUNEL+ population (Fig. 6C). Treatment with QVD at 10 µM inhibited B19V infection-induced TUNEL+ population by an efficiency of 70%. Similar to what was observed in the above transfection experiments, treatment with caspase-10 inhibitor showed a particularly high potency in inhibiting TUNEL+ population induced by B19V infection compared to treatments with other individual caspase inhibitors. At 20 µM, both caspase-10 inhibitor and Z-VAD treatments inhibited B19V infection-induced TUNEL+ population by more than 60%.
Collectively, our results show that caspase-10 inhibitor is the most effective inhibitor besides the two pan-caspase inhibitors in GFP-11kDa and GFP-NS1 transfection-induced apoptosis as well as in B19V infection-induced apoptosis.

**Caspase-10 is the most active caspase in 11kDa/NS1 transfection- and B19V infection-induced apoptosis.**

The proteolytic cleavage of poly(ADP-ribose)polymerase-1 (PARP1) is one of the hallmarks of apoptosis. The cleaved PARP1 band at a size of approximately 85 kDa was specifically detected in pGFP-NS1- and pGFP-11kDa-transfected UT7/Epo-S1 cells (Fig. 7A, lanes 5&6) as well as in B19V-infected CD36+ EPCs (Fig. 7A, lane 2), but not in GFP only transfected cells and mock cells (Fig. 4A, lanes 1, 3&4), supporting the apoptotic nature of cell death induced by transfection of the GFP-11kDa and the GFP-NS1 and by B19V infection. Moreover, the cleaved band of caspase-10 was specifically detected in the pGFP-NS1- and the pGFP-11kDa-transfected UT7/Epo-S1 cells (Fig. 7B, lanes 5&6, respectively), as well as in B19V-infected CD36+ EPCs (Fig. 7B, lane 2), but not in pGFP-transfected control cells (Fig. 7B, lane 4) and mock cells (Fig. 7B, lanes 1&3), suggesting that the caspase-10 is activated in 11kDa/NS1 transfection- and B19V infection-induced apoptosis.

Since caspase-3&7, -6 and -8 were reported active in NS1-expressing cell lines and B19V-infected erythroid progenitor cells, FLICA was employed to further evaluate the importance of the active caspase-10. As GFP and FAM share similar excitation and emission wavelength, we decided to transfet cells with RFP-11kDaHA, RFP-NS1HA and RFPHA (as a control). Cellular localization and expression level of these RFP fusion proteins were observed to be similar to that of GFP fusion proteins (data not shown). At 48 hrs posttransfection, RFP-NS1HA and RFP-11kDaHA activated caspase-10 in 24% and 48% of the transfected cells (αHA positive, HA+), respectively; in contrast, only 10% of RFPHA-expressing cells contained active caspase-10 (Fig. 7C). Similarly, activation of caspase-10 was detected in more than 53% of
B19V infected-CD36+ EPCs (capsid+) at 48 hrs p.i. (Fig. 7D). Not surprisingly, all other analyzed caspases (caspase-3&7, -6, -8 and -9) were also activated, however, at a much lower level, compared with caspase-10 in the RFP-NS1HA- and the RFP-11kDaHA-expressing cells (Fig. 7C) and in CD36+ EPCs infected with B19V (Fig. 7D). Our results show that caspase-10 is the most active caspase both in 11kDa- and NS1-expressing UT7/Epo-S1 cells and in B19V-infected CD36+ EPCs, and 11kDa is more efficient in activating caspase-10 than NS1.

**Discussion**

We report here for the first time that B19V 11kDa is a more significant inducer of apoptosis than NS1 during B19V infection of primary erythroid progenitor cells. B19V-11kDa–induced apoptosis is mediated by caspase-10 as an initiator. Strikingly, 11kDa expresses at least 100 times more than NS1 at the protein level during B19V infection of primary erythroid progenitor cells. In contrast with NS1, which localizes exclusively in the nucleus, 11kDa localizes predominately in cytoplasm, where apoptotic inducers usually reside. Although we used GFP-fused 11kDa and NS1 to analyze apoptosis induced in transfected cells, localization of the fused proteins recapitulates their native cellular localization. Moreover, the protein level of 11kDa in transfected cells was approximately 12 times lower than that during B19V infection of erythroid progenitor cells, while the level of the NS1 was comparable between transfection and infection. Therefore, the 11kDa-induced apoptosis by transfection closely reproduces apoptosis induced during B19V infection of primary erythroid progenitor cells. In addition, our results have shown that inhibition of 11kDa expression reduces apoptosis during B19V infection of CD36+ EPCs, and thus we conclude that the B19V 11kDa is the major functional protein in destroying erythroid progenitors during B19V infection.

Apoptosis is defined mechanistically as regulated cell death involving the sequential activation of caspases. Activation of caspase-8, -9 and -10, which are believed to be the initiator caspases at the top of the caspase signaling cascade, leads to the activation of downstream
caspases, including caspase-3, -6 and -7, which in turn induce apoptosis. As has been previously reported, caspase-3&7, -6 and -8 inhibitors can significantly reduce apoptosis induced by NS1 in established NS1-expressing cell lines as well as by B19V infection of erythroid progenitor cells, though at a high concentration of 200 µM. At this high concentration, we did observe more than 90% inhibition of cell death in both the transfection and infection system (data not shown). Instead of using such this “saturated” concentration, however, we applied the inhibitors at a low concentration (20 µM) to probe precisely the potency of the individual caspase inhibitor. Using FLICA, we detected a significantly higher level of the active caspase-10 than caspase-3&7, -6, -8 and -9 in 11kDa/NS1-transfection- and B19V-infection-induced apoptotic cells, strongly indicating that caspase-10 is the initiator caspase.

Caspase-10, previously considered as the ortholog of caspase-8, has been shown to be able to substitute the function of caspase-8. However, we found caspase-8 could not be substituted for caspase-10 in our study, as caspase-8 inhibitor was not nearly as effective as caspase-10 inhibitor in counteracting apoptosis induced by 11kDa and NS1 transfection in UT7/Epo-S1 and primary erythroid progenitor cells or induced during B19V infection of primary erythroid progenitor cells. In addition, the activation of caspase-10 is significantly higher than caspase-8 in both 11kDa- and NS1-transfected UT7/Epo-S1 cells and B19V-infected primary erythroid progenitor cells. This finding suggests a potential role of caspase-10 in the pro-apoptotic pathway that is not directly regulated by caspase-8.

Notably, B19V permissive cells are erythroid or magakaryoblastoid cells that require Epo to sustain differentiation and proliferation. Epo positively regulates erythropoiesis by preventing apoptosis and stimulating differentiation and proliferation of erythroid progenitors and erythroblasts. We observed that the amount of Epo, ranged from 0.1 to 10 units/ml in B19V infection of erythroid progenitor cells, did not influence the degree of apoptosis significantly (Fig. S1). On other hand, the Fas/Fas ligand pathway has been identified to have an apoptotic role in the regulation of erythropoiesis. Thus the balance between the apoptosis by the Fas/Fas
ligand and the antiapoptotic role in the presence of Epo is important for the homeostasis of erythroid progenitors. Since Epo strongly presents an antiapoptotic stimulation, apoptosis induced during B19V infection may require a high level of a potent inducer that is the 11kDa. We hypothesize that the high level of the 11kDa expression during B19V infection disturbs the balance between the apoptosis by the Fas/Fas ligand and the antiapoptotic role by Epo and further the homeostasis of erythroid progenitors. Thus, the B19V-11kDa-induced apoptosis provides us with a unique model to investigate further the mechanism underlying the caspase-10-dependent apoptosis, especially, in primary erythroid progenitor cells.

Although an infectious clone of B19V was established, progeny virus produced from transfection of this clone is apparently limited, and transfection of an 11kDa-knockout-clone only results in a few assembled particles exclusively localized in the nucleus. Therefore, we are unable to produce 11kDa-knockout virus to examine the role of the 11kDa in causing cell death of erythroid progenitors.

Direct cell death of infected erythroid progenitors results in the disease outcome of B19V infection. B19V must express abundant executors to erythroid progenitors during infection, among which the 11kDa is the most significant and abundant executor. Collectively, our data demonstrate that the B19V 11kDa protein is the major protein in executing erythroid progenitor cell death during B19V infection by inducing apoptotic cell death during B19V infection of erythroid progenitor cells that is mediated by activating caspase-10.

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Authorship
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References


**Figure legends**

**Fig. 1.** Transfection of 11kDa induces apoptosis in both B19V permissive and nonpermissive cells. (A&B) UT7/Epo-S1, CD36+ EPCs, HeLa, K562 and 293 cells were transfected with pGFP-11kDa plasmid (A) or pGFP as a control (B). CD36+ EPCs were stained
at 24 hrs posttransfection; other cells were stained at 48 hrs posttransfection with AnnexinV/PI double staining, followed by flow cytometry analysis. Both GFP negative [GFP(-)] and positive (GFP+) cell populations were gated to plot cells by PI vs AnnexinV. Only a representative experiment is shown, and the percentage of each quadrant is indicated. AnnexinV+ population is a combination of the AnnexinV+/PI+ population (number in the upper right quadrant) with the AnnexinV+/PI(-) population (number in the lower right quadrant). (C) The experiments as described in A&B were performed at least three times independently. The percentage value of AnnexinV+/PI+ or Annexin V+/PI(-), as shown in individual panel with indicated cell type, was calculated by subtracting the AnnexinV+/PI+ population or the AnnexinV+/PI(-) population of GFP+ cells by that of GFP(-) cells (background apoptosis). (D) UT7/Epo-S1 cells were transfected with pGFP-11kDa and stained with DAPI. Confocal images were taken at a magnification of 60 × (objective lens) with an Eclipse C1 Plus confocal microscope (Nikon). Arrows show apoptotic nuclei, which were enclosed in the apoptotic bodies visualized by GFP fluorescence. S1: UT7/Epo-S1; CD36*: CD36* EPCs; TX: transfection.

Results that are shown as average ± standard deviation in all the figures are generated from at least three independent experiments.

Fig. 2. Comparison of apoptosis induced by transfection of three B19V nonstructural proteins and by B19V infection. (A, B&C) Comparison of apoptosis induced by 7.5kDa, 11kDa and NS1 in UT7/Epo-S1 cells. UT7/Epo-S1 cells were transfected with pGFP control, pGFP-7.5kDa, pGFP-11kDa and pGFP-NS1. Cells were analyzed at the three time points of posttransfection as indicated. (A) The absolute percentages of AnnexinV+ populations of GFP+ cells were subtracted by that of GFP(-) cells and are plotted to the time points of posttransfection as shown. (B) UT7/Epo-S1 cells were transfected with plasmids expressing GFP or GFP-fused proteins as indicated. The mean fluorescence intensity (MFI) of GFP or GFP-fused proteins was detected by flow cytometer and plotted at three time points.
posttransfection. (C) The absolute values shown in panel A were normalized by the MFI of GFP shown in panel B that serves as a marker of protein expression level. The normalized data were plotted as relative values to GFP-11kDa, arbitrarily set as 100%. (D, E&F) Comparison of apoptosis induced by 7.5kDa, 11kDa and NS1 in CD36+ EPCs. The same plasmids, as indicated, were transfected to CD36+ EPCs. (D) The absolute percentages of AnnexinV+ populations of GFP+ cells were subtracted by that of GFP(-) cells and are plotted to the time points of posttransfection as shown. (E) CD36+ EPCs were transfected with plasmids expressing GFP or GFP-fused proteins as indicated. The mean fluorescence intensity (MFI) of GFP or GFP-fused proteins was detected by flow cytometer and plotted at 24 hrs posttransfection. (F) Results shown in panel D were normalized by following the same method used in panel C. (G) Apoptosis induced during B19V infection of CD36+ EPCs. The extent of apoptosis induced by mock/B19V infection of CD36+ EPCs was detected by TUNEL assay. Cells were also immunostained at the time points as indicated with an anti-B19V capsid antibody (clone 521-5D, Millipore) at 1:100 dilution followed by a FITC-conjugated secondary antibody with the TUNEL assay simultaneously. Stained cells were analyzed by flow cytometer, and both capsid+ and capsid(-) cell populations of B19V infected cells were gated for TUNEL+ population.

The symbols of a single star and double stars indicate P<0.05 and P<0.01, respectively.
S1: UT7/Epo-S1; CD36+: CD36+ EPCs; TX: transfection.

Fig. 3. Cellular localization and expression of 11kDa and NS1 in transfection.

(A) Specificity of αNS1 and α11kDa polyclonal antibodies. UT7/Epo-S1 cells transfected with pGFP-NS1 or pGFP-11kDa were stained with respective antisera followed by a Texas red-conjugated secondary antibody. Images were taken from an Eclipse SE TE2000-S UV microscope (Nikon) at a 20 × magnification. (B) Cellular localization of GFP-NS1 and GFP-11kDa in transfected UT7/Epo-S1 cells and CD36+ EPCs. UT7/Epo-S1 cells and CD36+
EPCs were transfected with pGFP-NS1 or pGFP-11kDa and stained with DAPI at 48 hrs posttransfection. DAPI was used to stain the nuclei. The confocal images (both panel B and C) were taken at 60 × (objective lens) magnification with an Eclipse C1 Plus confocal microscope (Nikon). (C) Cellular localization of 11kDa and NS1 in B19V-infected CD36+ EPCs. Infected CD36+ EPCs (at 48 hrs p.i.) were stained with α11kDa and αNS1 antisera followed by a Texas red-conjugated secondary antibody, respectively. DAPI was used to stain the nuclei. (D) The protein levels of GFP-NS1 and GFP-11kDa in transfected UT7/Epo-S1 cells and CD36+ EPCs vs the NS1 and the 11kDa expressed in B19V-infected CD36+ EPCs, respectively. UT7/Epo-S1 cells and CD36+ EPCs were transfected with either pGFP-11kDa or pGFP-NS1 and stained at 48 hrs posttransfection. CD36+ EPCs were infected with B19V and stained at 48 hrs p.i. Cells were fixed with 1% paraformaldehyde and permeabilized in 0.2% Tween-20. Either α11kDa or αNS1 antiserum at a dilution of 1:100 was used to immunostain cells, followed by a Cy5-conjugated secondary antibody. Stained cells were analyzed by flow cytometer. The protein level, represented by the mean fluorescence intensity, was compared between transfected and infected cells. S1: UT7/Epo-S1; CD36+: CD36+ EPCs; TX: transfection.

Fig. 4. Quantification of 11kDa and NS1 expression during B19V infection of CD36+ EPCs.
(A) Quantification of B19V 11kDa- and NS1-encoding mRNAs. CD36+ EPCs were infected with B19V. At 24, 48 and 72 hrs p.i., total RNA was isolated, treated with DNase, reverse-transcribed and quantified for absolute copies of mRNA by multiplex real-time PCR for NS1-mRNA/β-actin-mRNA and 11kDa-mRNA/β-actin-mRNA as described in Materials and Methods. The copy numbers of the 11kDa- and NS1-encoding mRNAs were normalized by copy numbers of β-actin mRNA in the same reaction and presented as numbers per copy of β-actin mRNA.

(B) Purity of purified fusion proteins GST-NS1(aa1-181) and GST-11kDa. Purified GST-NS1(aa1-181) and GST-11kDa proteins were resolved in SDS-10%PAGE gel and stained with Coomassie blue as shown. (C&D) Quantification of the steady-state protein level of 11kDa...
**vs NS1 during B19V infection.** 100 ng of GST-NS1(aa1-181) and 100 ng of GST-11kDa as seen in panel B and a serial dilution of them as shown were loaded in SDS-8%PAGE and SDS-15%PAGE for Western blot (panel C, lanes 1-7, and panel D, respectively). At 48 hrs p.i., 1.5 × 10^5 of CD36+ EPCs with or without (Mock) B19V infection were harvested, directly dissolved in SDS lysis buffer and loaded in lanes 6&7 (SDS-8%PAGE) and lanes 8&9 (SDS-6%PAGE) (panel C), and lanes 3&4 (SDS-15%PAGE) (panel D). Results from lanes as indicated also were quantified with Quantity One software (GE Health) and plotted to the right in panel C&D. Arrow and arrow head in panel C show NS1 specific band and GST-NS1(aa1-181), respectively; and arrow and arrow head in panel D show 11kDa specific band and GST-11kDa, respectively.

CD36+: CD36+ EPCs.

**Fig. 5. The inhibitory effects of 11kDa-specific Morpholinos on B19V-infection-induced apoptosis.** CD36+ EPCs were pre-treated with a control Morpholino (MO-Ctrl) or 11kDa-specific Morpholinos, MO-1, MO-2 and MO-3, as indicated, at a final concentration of 6 µM, 24 hrs prior to B19V infection. (A) A schematic diagram of the 11kDa-encoding mRNA and targets for specific Morpholino is shown. Regions in the 11kDa-encoding mRNA that Morpholinos target are shown with their respective nucleotide numbers. (B) Detection of B19V 11kDa protein. The same samples used for TUNEL assay were used for Western-blot using anti-11kDa antiserum. Detection of β-actin using a polyclonal antibody (ab1801, Abcam) served as a loading control. (C) TUNEL assay was performed with co-staining of B19V capsid using an anti-B19V capsid antibody (clone 521-5D, Millipore) for selection of infected cells at 48 hrs p.i. by flow cytometer. The TUNEL+ population is shown as a percentage in capsid positive cells.

**Fig. 6. The inhibitory effects of caspase inhibitors on 11kDa/NS1-transfection- and B19V-infection-induced apoptosis.** (A&B) Inhibitory effects of caspase inhibitors on apoptosis induced by 11kDa- and NS1-transfection. (A) UT7/Epo-S1 cells were transfected
with pGFP-11kDa or pGFP-NS1. (B) CD36+EPCs were transfected with pGFP-11kDa and pGFP-NS1, respectively, as shown. Individual caspase inhibitors (at 20 μM), caspase-1, -2, -3&7, -4, -6, -8, -9, -10, and -13 inhibitors, as indicated by 1, 2, 3&7, 4, 6, 8, 9, 10 and 13, and pan-caspase inhibitors, Z-VAD (20 μM) and Q-VD (10 μM) were applied at the time of transfection. DMSO served as a control at 0.5% v/v. Apoptosis was measured by AnnexinV/PI staining at different times posttransfection as indicated. The AnnexinV+/PI+ population is shown as a relative percentage (%) to the DMSO control that is arbitrarily set as 100%. (C) Inhibitory effects of caspase inhibitors on apoptosis induced by B19V infection. CD36+ EPCs were infected with B19V. Caspase inhibitors were applied upon infection at the concentrations described above. TUNEL assay was used to measure apoptosis induced in capsid+ cell population at 48 hrs p.i. by flow cytometer. The TUNEL+ population is shown as a relative % to the DMSO control, arbitrarily set as 100%.

All the numbers shown as percentage (%) are averages from at least two individual experiments.

Fig.7. PARP1 is cleaved and caspase-10 is the most active caspase in 11kDa/NS1-transfection- and B19V-infection-induced apoptosis. (A&B) Detection of cleaved PARP1 and cleaved caspase-10. CD36+EPCs were infected with B19V, and capsid+ cells were sorted at 48 hrs p.i. by flow cytometer. UT7/Epo-S1 cells were transfected with pGFP control, pGFP-11kDa and pGFP-NS1. At 48 hrs posttransfection, the GFP+ populations of transfected cells were sorted by flow cytometer. (A) Sorted cells were used for detecting the cleaved PARP1 by Western blot using anti-cleaved PARP1 at a dilution of 1:1000 (Cell Signaling). The blots were reprobed with anti-β-actin. (B) Sorted cells were used for detecting of active caspase-10 by Western blot using anti-caspase-10 at a dilution of 1:1000 (Sigma). Uninfected or pGFP transfected cells served as Mock as shown. The blots were reprobed with anti-β-actin. (C) Detection of activated caspase-3&7, -6, -8, -9 and -10 in 11kDa- and NS1- transfected cells.
UT7/Epo-S1 cells were transfected with pRFPHA control, pRFP-11kDaHA and pRFP-NS1HA. FAM-labeled FLICA peptides, FAM-DEVD-FMK, FAM-VEID-FMK, FAM-LETD-FMK, FAM-LEHD-FMK and FAM-AEVD-FMK, were used to detect active caspase-3/7, caspase-6, caspase-8, caspase-9 and caspase-10, respectively. Individual FLICA staining was performed to determine active caspases at 48 hrs posttransfection. Transfected cells were selected by intracellular staining of anti-HA tag at 1:100 dilution (clone HA-7, Sigma), shown as HA+ and HA(-) respectively, and were plotted to FLICA signal detected by flow cytometer. (D) Detection of activated caspase-3&7, -6, -8, -9 and -10 in B19V-infected CD36+ EPCs. CD36+ EPCs were infected with B19V. At 48 hrs p.i., cells were used for individual FLICA staining followed by intracellular staining with the antibody against B19V capsid. Both capsid positive and negative cells were plotted to FLICA signal detected by flow cytometer. TX: transfection.
Figure 1
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Figure 7
The small 11kDa non-structural protein of human parvovirus B19 plays a key role in inducing apoptosis during B19 virus infection of primary erythroid progenitor cells

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