Hantaviruses induce cytopathic effects and apoptosis in continuous human embryonic kidney cells

A. Markotic, 1 L. Hensley, 2 T. Geisbert, 2 K. Spik 2 and C. Schmaljohn 2

1Department for Research and Development, Institute of Immunology, Zagreb, Croatia
2Virology Division, United States Army Medical Research Institute of Infectious Diseases, 1301 Ditto Ave, Fort Detrick, MD 21702, USA

Hantaviruses are maintained in nature in persistently infected rodents and can also persistently infect cultured mammalian cells, causing little or no cytopathology. An unexpected outcome of this study was the observation of cytopathic effects (CPE) in the hantavirus-infected human embryonic kidney cell line HEK293. It was confirmed that hantaviruses induce apoptosis in HEK293 cells, although apoptosis appeared mostly in uninfected, bystander cells and rarely in infected HEK293 cells. Although studies by others suggest that the nucleocapsid protein of Puumala virus interacts with the Fas-mediated apoptosis enhancer Daxx at the gene expression level, it was determined that members of the TNF receptor superfamily did not contribute to the apoptosis observed in infected HEK293 cells. The observation of CPE in HEK293 cells might lead to a better understanding of the mechanisms of persistence and pathogenesis in hantavirus infections.

The genus Hantavirus includes several of the most significant human pathogens belonging to the family Bunyaviridae. Hantaviruses cause two serious human diseases: haemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (reviewed by Schmaljohn & Hjelle, 1997). Immunopathogenesis is suggested to be involved in both HFRS and HPS (reviewed by Markotic et al., 1999). A common feature of hantavirus diseases is increased permeability in microvascular beds, suggesting that the vascular endothelium is a prime target for virus infection. Endothelial cells are susceptible to hantavirus infection; however, virus does not cause cytopathic effects (CPE) to explain the increase in endothelial permeability (reviewed by Khaiboullina & St Jeor, 2002).

Generally, hantaviruses are maintained in nature in persistently infected rodents and can also persistently infect cultured mammalian cells, causing little or no cytopathology (reviewed by Meyer & Schmaljohn, 2000). Two reports described low pH-dependent cell fusion that occurred in cultured cells infected with hantaviruses (Arikawa et al., 1985; McCaughey et al., 1999). An unexpected outcome of this study was our observation of CPE in the infected human embryonic kidney cell line HEK293. To our knowledge, it is the first report of frank cytopathogenicity due to infection by a hantavirus in any cell line.

The following viruses were used to infect HEK293 cells (ATCC CRL 1573) (Graham et al., 1977): Hantaan virus (HTNV), strain 76118 (Lee et al., 1978); Seoul virus (SEOV), strain SR-11 (Kitamura et al., 1983); Andes virus (ANDV), strain Chile-9717869 (Toro et al., 1998); Sin Nombre virus (SNV), strain CC107 (Schmaljohn et al., 1995); and Laguna Negra virus (LNV), strain AF005727 (Johnson et al., 1997). All viruses were propagated in Vero E6 cells (Vero C1008, ATCC CRL 1586). All infected and non-infected cells were tested for mycoplasma contamination by ELISA-PCR (Roche) and were found to be mycoplasma free. Subconfluent HEK293 cells (3 days after seeding) were infected at an m.o.i. of 0·1 p.f.u. per cell (SNV), 1 p.f.u. per cell (ANDV) or 10 p.f.u per cell (HTNV). As controls for the infected cultures, mock infections of HEK293 cells were performed using the same medium that we used for diluting the viruses prior to adsorption. That is, for mock infections, we added fresh adsorbing medium (MEM with 10 % FBS) to cell monolayers and incubated the cells under the same conditions as the infected cells for which they were serving as controls. The cells were incubated for 8–14 days at 37 °C and 5 % CO2 and medium was not changed during the entire period of observation. Cultures were examined daily under an inverted microscope and the resulting CPE was noted. At 11 days after infection, cells were fixed with methanol and stained with Wright–Giemsa stain. This stain, which contains polychrome methylene blue and eosin Y, is used traditionally for staining blood smears, parasites and virus inclusions. We used it in our studies to provide better resolution of cellular detail (not shown).

CPE could be detected as early as 3–4 days after infection with HTNV, SEOV or ANDV. By days 10–11 after infection, all of the hantavirus-infected cultures, except those infected with SNV and LNV, showed extensive CPE. SNV and LNV
A. Markotic and others

A

<table>
<thead>
<tr>
<th></th>
<th>Native</th>
<th>IFA</th>
<th>TUNEL assay + IFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td><img src="image1" alt="Mock" /></td>
<td><img src="image2" alt="Mock IFA" /></td>
<td><img src="image3" alt="Mock TUNEL" /></td>
</tr>
<tr>
<td>HTNV</td>
<td><img src="image4" alt="HTNV" /></td>
<td><img src="image5" alt="HTNV IFA" /></td>
<td><img src="image6" alt="HTNV TUNEL" /></td>
</tr>
<tr>
<td>SEOV</td>
<td><img src="image7" alt="SEOV" /></td>
<td><img src="image8" alt="SEOV IFA" /></td>
<td><img src="image9" alt="SEOV TUNEL" /></td>
</tr>
<tr>
<td>ANDV</td>
<td><img src="image10" alt="ANDV" /></td>
<td><img src="image11" alt="ANDV IFA" /></td>
<td><img src="image12" alt="ANDV TUNEL" /></td>
</tr>
</tbody>
</table>

B

![Box plot](image13)
required 12–14 days to cause strong CPE in HEK293 cells. No CPE was observed in mock-infected cultures. Reproducible and convincing CPE could be observed only in cells that were in good condition when they were infected and which had been propagated no more than 8–10 times after thawing. Generally, we observed that a higher m.o.i., i.e. higher concentrations of virus (>10^6 p.f.u. ml\(^{-1}\)), gave clear, strong CPE with obvious cell destruction (data not shown). After day 10, it was possible to observe CPE in cells infected at a low m.o.i., i.e. concentrations of 10^2–10^3 p.f.u. ml\(^{-1}\) (data not shown). Consequently, it is likely that the slower development of CPE in cells infected with SNV or LNV was due to our inability to achieve a high m.o.i. because of low-titre seed stocks of viruses.

To confirm the infection of cells, we performed immunofluorescent antibody (IFA) tests (Fig. 1A). Primary specific monoclonal antibodies to the G2 protein of HTNV (8E10) (Arikawa et al., 1989) or hyperimmune mouse ascites fluid (HMAF) to the specified hantaviruses were used for staining.

To verify that virus replication was occurring and to examine virus growth characteristics in the HEK293 cells, we measured infectious virus in supernatants of cells infected with one HFRS-causing virus (HTNV) or one HPS-causing virus (ANDV) at different times after infection. Plaque assays were performed as described previously (Hooper et al., 2001). Infectious virus was detected at 6–20 h after infection, with peak amounts observed between days 3 and 4 after infection. Titres started to decline by 5 days after infection and rapidly decreased until 8 days after infection, coincident with CPE (data not shown).

At least two general pathways are known to lead to cell death and CPE: necrosis and apoptosis. Many viruses cause apoptosis, or programmed cell death, which is an important mechanism for eliminating virus-infected cells from the host (reviewed by O’Brien, 1998). Hantaviruses replicate in vitro in the cytoplasm of a variety of mammalian cell lines, but also in primary endothelial cells and monocyte/macrophages, and do not cause visible CPE (reviewed by Kanerva et al., 1998). Apoptosis has been described for HTNV-infected Vero E6 cells in the absence of CPE (Kang et al., 1999). To determine if the CPE that we observed in HEK293 cells was caused by apoptosis or necrosis, we performed TUNEL (transferase-mediated deoxyuridine triphosphate nick-end labelling) assays in cells infected with HTNV, ANDV and SEOV (Fig. 1A). TUNEL stains were performed using the ApopTag Red assay (Intergen), according to the manufacturer’s recommendations with minor modifications. To determine if virus infection was related directly to apoptosis, IFA for viral antigen was performed after the last step of the TUNEL procedure. Staining was visualized using appropriate single-pass and triple-pass filters. The number of apoptotic cells was measured by counting the number of TUNEL-positive cells per random field, with a minimum of five fields counted. Although occasional cells showed both apoptosis and hantaviral antigen, most of the apoptotic cells did not have detectable hantaviral antigen but, instead, were adjacent to cells with viral antigen (i.e. bystander cells) (Fig. 1A, TUNEL assay + IFA). All cells, except those infected with HTNV at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\), had significantly more apoptotic cells than mock-infected HEK293 cells (Fig. 1B). Also, cells infected with hantaviruses at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\) had significantly more apoptotic cells than cells infected at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\) (Fig. 1B). Finally, although at an m.o.i. of 1 p.f.u. ml\(^{-1}\), SEOV induced the highest number of apoptotic cells, there were no statistically significant differences among the viruses tested (Fig. 1B). For the analysis of variance, a multiple comparison procedure was used (Tukey’s HSD test) (Tukey, 1949).

Because single-stranded DNA ends can be detected in necrotic cells, causing false-positive signals (Collins et al., 1997), we employed transmission electron microscopy (TEM) in conjunction with TUNEL to confirm apoptosis in cells infected with HTNV, ANDV and SEOV. Cultures of infected or mock-infected HEK293 cells were processed for TEM by conventional methods, as described previously (Hensley et al., 2002). Briefly, cells were fixed in 2 % glutaraldehyde in 0·1 M phosphate buffer, post-fixed in 1 % osmium tetroxide, rinsed, treated with 0·5 % uranyl acetate in ethanol, dehydrated in ethanol and propylene oxide and embedded in Poly/Bed 812 resin (Polysciences). Ultrathin sections were cut, placed on 200-mesh copper TEM grids, stained with uranyl acetate and lead citrate and examined under a JEOL 1200 EX TEM (JEOL). More than 60 fields were examined using triple-pass filters. The number of apoptotic cells was measured by counting the number of TUNEL-positive cells per random field, with a minimum of five fields counted. Although occasional cells showed both apoptosis and hantaviral antigen, most of the apoptotic cells did not have detectable hantaviral antigen but, instead, were adjacent to cells with viral antigen (i.e. bystander cells) (Fig. 1A, TUNEL assay + IFA). All cells, except those infected with HTNV at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\), had significantly more apoptotic cells than mock-infected HEK293 cells (Fig. 1B). Also, cells infected with hantaviruses at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\) had significantly more apoptotic cells than cells infected at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\) (Fig. 1B). Finally, although at an m.o.i. of 1 p.f.u. ml\(^{-1}\), SEOV induced the highest number of apoptotic cells, there were no statistically significant differences among the viruses tested (Fig. 1B). For the analysis of variance, a multiple comparison procedure was used (Tukey’s HSD test) (Tukey, 1949).

Because single-stranded DNA ends can be detected in necrotic cells, causing false-positive signals (Collins et al., 1997), we employed transmission electron microscopy (TEM) in conjunction with TUNEL to confirm apoptosis in cells infected with HTNV, ANDV and SEOV. Cultures of infected or mock-infected HEK293 cells were processed for TEM by conventional methods, as described previously (Hensley et al., 2002). Briefly, cells were fixed in 2 % glutaraldehyde in 0·1 M phosphate buffer, post-fixed in 1 % osmium tetroxide, rinsed, treated with 0·5 % uranyl acetate in ethanol, dehydrated in ethanol and propylene oxide and embedded in Poly/Bed 812 resin (Polysciences). Ultrathin sections were cut, placed on 200-mesh copper TEM grids, stained with uranyl acetate and lead citrate and examined under a JEOL 1200 EX TEM (JEOL). More than 60 fields were examined using triple-pass filters. The number of apoptotic cells was measured by counting the number of TUNEL-positive cells per random field, with a minimum of five fields counted. Although occasional cells showed both apoptosis and hantaviral antigen, most of the apoptotic cells did not have detectable hantaviral antigen but, instead, were adjacent to cells with viral antigen (i.e. bystander cells) (Fig. 1A, TUNEL assay + IFA). All cells, except those infected with HTNV at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\), had significantly more apoptotic cells than mock-infected HEK293 cells (Fig. 1B). Also, cells infected with hantaviruses at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\) had significantly more apoptotic cells than cells infected at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\) (Fig. 1B). Finally, although at an m.o.i. of 1 p.f.u. ml\(^{-1}\), SEOV induced the highest number of apoptotic cells, there were no statistically significant differences among the viruses tested (Fig. 1B). For the analysis of variance, a multiple comparison procedure was used (Tukey’s HSD test) (Tukey, 1949).

Because single-stranded DNA ends can be detected in necrotic cells, causing false-positive signals (Collins et al., 1997), we employed transmission electron microscopy (TEM) in conjunction with TUNEL to confirm apoptosis in cells infected with HTNV, ANDV and SEOV. Cultures of infected or mock-infected HEK293 cells were processed for TEM by conventional methods, as described previously (Hensley et al., 2002). Briefly, cells were fixed in 2 % glutaraldehyde in 0·1 M phosphate buffer, post-fixed in 1 % osmium tetroxide, rinsed, treated with 0·5 % uranyl acetate in ethanol, dehydrated in ethanol and propylene oxide and embedded in Poly/Bed 812 resin (Polysciences). Ultrathin sections were cut, placed on 200-mesh copper TEM grids, stained with uranyl acetate and lead citrate and examined under a JEOL 1200 EX TEM (JEOL). More than 60 fields were examined using triple-pass filters. The number of apoptotic cells was measured by counting the number of TUNEL-positive cells per random field, with a minimum of five fields counted. Although occasional cells showed both apoptosis and hantaviral antigen, most of the apoptotic cells did not have detectable hantaviral antigen but, instead, were adjacent to cells with viral antigen (i.e. bystander cells) (Fig. 1A, TUNEL assay + IFA). All cells, except those infected with HTNV at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\), had significantly more apoptotic cells than mock-infected HEK293 cells (Fig. 1B). Also, cells infected with hantaviruses at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\) had significantly more apoptotic cells than cells infected at an m.o.i. of 0·1 p.f.u. ml\(^{-1}\) (Fig. 1B). Finally, although at an m.o.i. of 1 p.f.u. ml\(^{-1}\), SEOV induced the highest number of apoptotic cells, there were no statistically significant differences among the viruses tested (Fig. 1B). For the analysis of variance, a multiple comparison procedure was used (Tukey’s HSD test) (Tukey, 1949).
per culture were examined (more than 30 fields from two different sections taken from different areas/levels of the cell pellet). At a magnification of ×2000, each field contained between 4 and 11 cellular nuclei, of which one to three appeared apoptotic. In contrast, apoptotic cells were rarely seen in the mock-infected cultures. Cells representing various stages of apoptosis were observed in all cultures infected (Fig. 2); most prominent were large apoptotic bodies in the cytoplasm of otherwise normal HEK293 cells, indicating phagocytosis of apoptotic bodies by neighbouring cells (Fig. 2).

One possible mechanism for induction of apoptosis could be mediated by TNF and members of the TNF superfamily (reviewed by O’Brien, 1998). In a previous study using HEK293 cells, apoptosis was found to be mediated by FasL-induced Fas/Apo1 (Larregina et al., 1998). Recently, the nucleocapsid protein of another HFRS-causing hantavirus, Puumala virus, was reported to interact with the Fas-mediated apoptosis enhancer Daxx (Li et al., 2002). Consequently, we investigated the possibility that the TNF receptor superfamily (which includes FasL) contributed to the apoptosis that we observed. For that purpose, we measured the mRNA levels of FasL, Fas, FADD, DR3, FAP, FAF, TRAIL, caspase-8, TNFRp55, TRADD and RIP using a ribonuclease protection assay. Total HEK293 RNA was extracted using Trizol reagent (Life Technologies), followed by phase separation with chloroform and precipitation with isopropyl alcohol. Apoptosis transcripts were measured using a RiboQuant MultiProbe RNase Protection system (PharMingen) and multi-probe template set (hAPO-3), following the instructions provided by the manufacturer.

Quantitative comparisons of the levels of mRNA expression among samples were made using a CYCLONE storage phosphorimaging system (Packard Instrument Company) with molecular analysis software (OPTIQUANT, version 3.0). The values obtained for each level of mRNA measured were normalized against the combined levels of expression obtained for mRNA from GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and L32 housekeeping genes loaded within the same lane on the ribonuclease protection assay gel. We did not find any statistical differences among molecules responsible for varying mechanisms of death-inducing functions in the TNF superfamily between infected and non-infected cells. Only a slight increase in TNFRp55 could be seen but, using image analysis, no statistical differences between infected and non-infected cells were found (Fig. 3).

In our study, we found that hantaviruses caused CPE and induced apoptosis in HEK293 cells. However, at the gene expression level, we did not find that the TNF receptor superfamily were responsible for it. We also observed that apoptosis appeared mostly in uninfected, bystander cells and rarely in infected HEK293 cells. These findings suggest that apoptosis induction may occur independently of hantavirus replication and that production of some soluble factors may contribute to apoptosis. The appearance of bystander apoptosis does not necessarily rule out a direct mechanism or multiple mechanisms of apoptosis by the virus, as apoptosis was seen in some infected cells as well. For instance, hantavirus infection might induce nutrient starvation in the infected cells, leading to apoptosis (reviewed by Everett & McFadden, 1999), or potentially...
Hantaviruses induce apoptosis in HEK293 cells

Fig. 3. Ribonuclease protection assay showed no obvious differences in the expression of mRNA levels of FasL, Fas, FADD, DR3, FAP, FAF, TRAIL, caspase-8, TNFRp55, TRADD and RIP in HEK293 cells infected with HTNV, SNV or ANDV in comparison to non-infected (mock) cells at 5 days after infection. Only a slight increase in TNFRp55 could be seen but, using image analysis, no statistical differences between infected and non-infected cells were found.

Our finding of CPE in HEK293 cells, despite the absence of CPE in other cells, is not without precedent. HEK293 cells support the replication of the fastidious enteric adenovirus serotypes 40 and 41, as well as a variety of other viruses, such as herpes simplex virus, parainfluenza viruses, respiratory syncytial virus and enteroviruses. All of them cause varying degrees of CPE in HEK293 cells but not in other cell lines (Brown & Petric, 1986).

One possible factor of the ability of hantaviruses to induce CPE in HEK293 cells may be the presence of a vitronectin receptor, an \( \alpha_2\beta_1 \) integrin heterodimer that is present on the surface of HEK293 cells (Bodary & McLean, 1990). The \( \alpha_2\beta_1 \) integrins, also vitronectin receptors, are receptors for pathogenic hantaviruses (Gavrilovskaya et al., 1998). They are known for transduction of intracellular signals, promoting survival of endothelial cells and various tumour cells, which are adhesion dependent (reviewed by Brassard et al., 1999). Also, treatment with the \( \alpha_2\beta_1 \) antagonists result in an apoptotic response (reviewed by Brassard et al., 1999). However, the possible role of integrins in HTNV-induced cytopathology in HEK293 has to be determined.

Our observation of CPE in HEK293 cells might lead to a better understanding of the mechanisms of persistence and pathogenesis in hantavirus infections.

ACKNOWLEDGEMENTS

We thank Dr Ante Sabioncello for his kind assistance in statistical analysis of our data. These studies were performed while Dr A. Markotic held a National Research Council fellowship at USAMRIID.

REFERENCES


