Hantaan virus nucleocapsid protein stimulates MDM2-dependent p53 degradation

Sun-Whan Park,1,2 Myung-Guk Han,1 Chan Park,1 Young Ran Ju,3 Byung-Yoon Ahn2 and Jungsang Ryou1

Correspondence
Jungsang Ryou
zenith@nih.go.kr

1Division of Arboviruses, Center for Immunology & Pathology, National Institute of Health, Korea Centers for Disease Control & Prevention, Republic of Korea
2School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Republic of Korea
3Division of Zoonoses, Center for Immunology & Pathology, National Institute of Health, Korea Centers for Disease Control & Prevention, Republic of Korea

Apoptosis has been shown to be induced and downregulated by the Hantaan virus (HTNV) nucleocapsid (N) protein. To address these conflicting data, expression of the p53 protein, one of the key molecules involved in apoptosis, was assessed in the presence of the N protein in A549 and HeLa cells. The amount of p53, increased by drug treatment, was reduced when cells were infected with HTNV or transfected with an expression vector of the HTNV N protein. When cells were treated with a proteasome inhibitor (MG132) or an MDM2 antagonist (Nutlin-3), p53 expression was not reduced in N protein-overexpressed cells. We concluded that the HTNV N protein ubiquitinates and degrades p53 MDM2-dependently. Here we report downregulation of p53 expression through a post-translational mechanism: MDM2-dependent ubiquitination and degradation by the HTNV N protein. These results indicate that N protein-dependent p53 degradation through the ubiquitin proteasome system is one of the anti-apoptotic mechanisms employed by HTNV.

Viruses of the genus *Hantavirus* (family *Bunyaviridae*) have three segmented negative sense RNAs as their genome. The N protein, encoded by the S segment, of Hantaan virus (HTNV) contains 429–433 amino acid residues and has a molecular mass of approximately 50 kDa. It is almost twice as long as the N protein in viruses of other genera of *Bunyaviridae* (26–29 kDa) (van Regenmortel et al., 2000). This N protein binds, forms ribonucleoproteins and encapsidates the genomic RNAs into the three viral segments (Mir & Panganiban, 2004; Severson et al., 2001). It is the most abundant viral protein in infected cells, where it is located in the cytoplasm and forms inclusion bodies and filamentous structures (van Regenmortel et al., 2000). It is multifunctional, involved in various interactions with cellular components during the life cycle of the virus. It has important functions in viral RNA replication and assembly (van Regenmortel et al., 2000). A primary function of the viral N protein is to protect the RNA genome (Kinpe et al., 2001).

When hantaviruses infect cells, the N protein interacts with several cellular proteins. Thus, the N protein of Puumala virus interacted with Daxx (Li et al., 2002), ubc9, and small ubiquitin-like modifier-1 (SUMO-1) (Kaukinen et al., 2003). It was also reported that ubc9 and SUMO-1 interacted with the N protein of HTNV in a yeast and mammalian two-hybrid system (Maeda et al., 2003). The N proteins of HTNV and Seoul virus (SEOV) interacted with SUMO-1, ubc9, protein inhibitor of activated signal transducers and activators of transcription (PIAS), homeodomain-interacting protein kinase 2 (HIPK2), chromodomain helicase DNA-binding protein 3 (CHD3), TRAF and TNF receptor-associated protein (TRRAP), and Ran-binding protein in microtubule-organizing centre (RanBPM) (Lee et al., 2003). p53 is stabilized and activated by various cellular stresses such as DNA damage, heat shock, hypoxia, and endoplasmic reticulum stress, leading to growth arrest or apoptosis (Vogelstein et al., 2000). Proapoptotic activity of p53 is both transcription-dependent and independent. Its amount and activity are regulated by specific post-translational modifications including phosphorylation, acetylation, ubiquitination, sumoylation, and protein–protein interactions (Ferecatu et al., 2009).

p53 has a very short half-life in normal cells, whereas its half-life is dramatically prolonged in human tumour cells (Rotter, 1983). MDM2 is the primary negative regulator of p53. The tumorigenic potential of MDM2 was closely linked to its repressive function with respect to the p53 tumour suppressor gene. Physical association was detected
between the p53 and MDM2 proteins through immunoprecipitation studies (Hinds et al., 1987). The relationship between MDM2 and p53 is not unidirectional. Despite the negative regulation of p53 by MDM2, p53 positively regulates MDM2 by acting as a transcription factor at the mdm2 gene. Therefore, the two way relationship between MDM2 and p53 forms an autoregulatory negative feedback loop. The balance between the two proteins maintains a low cellular level of p53 and limits the duration and potency of the p53 response during stress.

There are several reports on the association of hantaviruses with apoptosis. Some viruses, including HTNV, Andes virus, SEOV, and Prospect Hill virus, produced apoptosis in cells following infection (Kang et al., 1999; Markotic et al., 2003; Nam et al., 2003). Some evidence of apoptosis such as DNA fragmentation, caspase activation and poly ADP ribose polymerase cleavage is evident in cells infected with HTNV and Tula (TULV) hantaviruses (Kang et al., 1999; Li et al., 2004, 2005). The presence of apoptotic cells was reported following in vivo infection by hantaviruses in humans (Klingström et al., 2006) and hamsters (Schönrich et al., 2008). In a recent report, modulation of apoptosis appeared different between pathogenic and non-pathogenic hantaviruses; the non-pathogenic TULV induced apoptosis in the host cells but the pathogenic HTNV did not (Li et al., 2004, 2005). The full-length N protein of HTNV inhibited apoptosis while a truncated version did not (Ontiveros et al., 2010). It was also reported that there were many differences in the immune responses in organisms infected with pathogenic and non-pathogenic hantaviruses (Alff et al., 2006; Rang, 2010; Shim et al., 2011). These reports indicate the existence of differences in the molecular mechanisms in cells infected with pathogenic hantavirus forms compared with non-pathogenic hantavirus forms.

In this study, we elucidated that the HTNV N protein is capable of downregulation of p53 levels through a post-translational mechanism: degradation after MDM2-dependent ubiquitination of the protein. This down-regulation of p53 might be associated with the inhibition of apoptosis in haemorrhagic fever with renal syndrome (HFRS)-inducing hantaviruses. These results could provide beneficial information for further studies to elucidate HFRS pathogenesis.

In order to estimate the influence of HTNV on p53 expression in infected cells, we assayed p53 by Western blot analysis. Cells were harvested and lysed with NP-40 cell lysis buffer (Invitrogen) after HTNV infection and with drug treatment or without, according to experiments. Total proteins were separated and blotted onto PVDF membranes. They were then probed with a mouse monoclonal anti-p53 (DO-1) (Santa Cruz). A rabbit polyclonal anti-GAPDH (Santa Cruz) was used as a protein-loading control. First we monitored changes in N protein and p53 expression profiles after HTNV infection in A549 cells without drug treatments (Fig. S1, available in JGV Online).

HTNV N protein expression increased over 4 days post-infection and the p53 level was inversely proportional to that of the N protein. At day 4, minimal levels of p53 were expressed when the N protein was highly expressed. We also artificially induced cellular p53 by treatment with apoptosis-inducing drugs, doxorubicin (0.8–3.2 mmol l$^{-1}$) or cisplatin (5–80 mmol l$^{-1}$) after HTNV infection in HeLa cells, to mimic the apoptosis-inducing effects of mediators that may be produced by HTNV infection. The p53 level in HeLa cells was increased after treatment with doxorubicin or cisplatin in a dose-dependent manner (Fig. 1a). We compared the amount of p53 in HTNV-infected cells and non-infected cells after inducing apoptosis by treatment with doxorubicin and cisplatin. HTNV infection was confirmed by probing with a primary polyclonal anti-N protein antibody (prepared in mice). The amount of p53 was decreased when cells were infected with HTNV (Fig. 1b).

To confirm that inhibition of p53 expression by HTNV infection is caused by the N protein, we overexpressed the N protein in HeLa and A549 cells (Fig. 2). At first, we constructed a mammalian cell expression plasmid by inserting the open reading frame of the S segment of HTNV to the vector ‘pcDNA3.1/His B’ (Invitrogen) (Fig. 2a). To monitor N protein expression in cells, immunofluorescence assay

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**Fig. 1. Reduction of drug-induced p53 levels by HTNV.** (a) Induction of p53 expression in HeLa cells following treatment with doxorubicin (0.8, 1.6, 2.4 and 3.2 mmol l$^{-1}$) or cisplatin (5, 10, 20, 40 and 80 mmol l$^{-1}$). Western blot analyses showed the level of p53 increased gradually in cells dose-dependently. (b) Western blot analysis of p53 in HeLa cells treated with doxorubicin and cisplatin after infection with HTNV (m.o.i. of 5). HeLa cells were treated with cisplatin and doxorubicin at 4 days post-infection and 24 h later cell extracts were immunoblotted with anti-p53 or anti-HTNV N protein antibodies. Anti-GAPDH was used as a protein-loading control.
(IFA) was carried out using a polyclonal antibody against the N protein after transfection of Vero-E6 cells with the N protein-expressing plasmid (pcHNP). In this study, the polyclonal antibody against HTNV was used as the primary antibody, and Hoechst dye (Molecular Probes) and Texas red or FITC-labelled anti-mouse antibody (Vector Laboratories) was used to stain chromatin and the HTNV N protein, respectively. The fluorescence microscopic image showed N protein was expressed in the cells and localized in the perinuclear area (Fig. 2b), with a similar distribution in HTNV-infected cells. We induced p53 by treatment of cells with cisplatin (40 mmol l$^{-1}$) or doxorubicin (3.2 mmol l$^{-1}$) 1 day after pcHNP transfection of cells. At 2 days after transfection, we harvested cell lysates and analysed the p53 level. In mock-transfected control cells, p53 was increased by treatment with the apoptosis-inducing drugs. On the other hand, p53 was decreased in the N protein-overexpressing cells. Thus, the N protein of HTNV is the molecule causing the p53 downregulation by HTNV shown in Fig. 1.

To elucidate the mechanism of p53 downregulation by HTNV N protein, we first estimated the p53 mRNA level of drug-treated cells after HTNV infection by reverse transcription-PCR, with the result indicating that HTNV did not influence p53 gene transcription (data not shown). Thus, we concluded that the downregulation of p53 by HTNV was post-transcriptional.

p53 is mainly regulated by its degradation post-translationally. We assessed p53 half-life following infection by the use of a protein synthesis inhibitor, cycloheximide (CHX), and quantifying p53 levels. However, we did not obtain the expected result because p53 was already reduced to minimum levels in the N protein-expressing conditions (Fig. S2a). MDM2 is the main regulator of p53 degradation through the ubiquitin-dependent proteasome system. The involvement of ubiquitination and MDM2 in the inhibition of p53 expression by HTNV was assayed by exposing cells to 6 μmol MG132 l$^{-1}$, a proteasome inhibitor, or 10 μmol Nutlin-3 l$^{-1}$, an MDM2 antagonist, for 18 h. Both

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**Fig. 2.** Reduction of drug-induced p53 levels by the HTNV N protein. (a) Map of the vector, pcHXHNP. This contains the CMV and T7 promoters and uses His or Xpress as a tag. (b) Confocal microscopic image of cells in which HTNV N proteins were expressed by HTNV infection or transfection with pcHXHNP. The nucleus (blue) was stained with Hoechst dye. Transfected N protein was detected with a rabbit anti-His antibody (red). Infected N protein was detected using a mouse anti-HTNV antibody (green) as a control. Original magnification, ×400. (c, d) Western blot analyses of p53 expression levels were performed after HeLa (c) and A549 (d) cells were transiently transfected with 3 μg pcHXHNP and treated with cisplatin (40 mmol l$^{-1}$) or doxorubicin (3.2 mmol l$^{-1}$) 24 h later. An anti-GAPDH antibody was used as a protein-loading control.
MG132 and Nutlin-3 entirely restored the p53 levels that had been decreased by HTNV infection or N protein overexpression with drug treatment or without (Figs 3a, b, S2b). The involvement of MDM2 in p53 regulation by the N protein was confirmed by co-expressing N protein and dominant negative MDM2 (Fig. S3). Dominant negative MDM2 restored the p53 level which was decreased by N protein overexpression. Thus, the HTNV N protein induced degradation of p53 by a mechanism involving the action of MDM2. Ubiquitination of p53 was also detected by immunoprecipitation with anti-p53 and Western blotting using an anti-ubiquitination antibody (Cell Signalling Technologies) (Fig. 3c). HeLa cells were pretreated with lactacystin (1 μmol l⁻¹) for 24 h before immunoprecipitation. Ubiquitination of p53 was reduced by cisplatin and doxorubicin (lanes 2 and 3) in non-infected HeLa cells, but not in HTNV-infected cells (lanes 5 and 6). These results indicate that HTNV induces p53 polyubiquitination, which is inhibited during apoptosis of cells.

There is as yet limited information on the biological functions of the hantavirus gene products to explain the molecular mechanisms that trigger and control the presumed pathogenesis of HFRS in infected tissues. For example, elevated levels of T-cell inflammatory cytokines, such as TNF-α, are considered to be important markers in the pathogenesis of HFRS. We hypothesized that direct interactions of hantavirus gene products with host cell proteins might be central in this process. The N protein is the major structural component of hantaviruses and interacts with various cellular proteins such as actin microfilaments, Daxx and proteins acting in sumoylation such as PIAS1, PIASxβ, TTRAP, CHD3 and HIPK2 (Lee et al., 2003). Among them, HIPK2 interacts with several transcription factors that have important biological functions. It plays a pivotal role in p53-mediated apoptosis through phosphorylation at its Ser46 position. Our study was primarily intended to focus on the interaction between N protein and HIPK2 and its effect on cellular responses including apoptosis of HTNV-infected cells. But a quantitative, not qualitative (phosphorylation), change in p53 in cells infected by HTNV was discovered in the study as shown in Fig. 1.

When humans are infected with viruses, many cytokines including TNF-α are produced by immune cells triggered in turn by virus-infected cells. The cytokines can stimulate cells to die, but HTNV-infected cells resist dying and continue to stimulate immune cells. The accumulated mediators can induce organ damage locally such as nephropathy and/or systemically, for example by haemorrhage. Therefore, we intentionally induced apoptosis by treatment of cells with doxorubicin or cisplatin to mimic the effect of cytokines in human HFRS. Intriguingly, when apoptosis was induced by drug treatment, the amount of p53 was dramatically decreased in the HTNV-infected cells compared with the non-infected cells. We demonstrated that the N protein is the molecule causing p53 down-regulation in HTNV-infected cells. p53 was downregulated in cells infected with HTNV or overexpressing the N protein. To characterize the mechanism of p53 down-regulation, firstly the p53 mRNA level was estimated, but p53 transcript was not reduced by HTNV. p53 levels following MG132 or Nutlin-3 treatment were also assessed.
and to confirm the results we detected ubiquitinated p53 protein with anti-ubiquitin antibody after immunoprecipitation with anti-p53. From these two experiments, we concluded protein ubiquitination was involved in p53 downregulation by HTNV.

In this study, we confirmed the inhibition of apoptosis by HTNV infection and elucidated a molecular mechanism of how p53 expression is downregulated by the HTNV N protein. From these results we propose a molecular mechanism of HFRS as follows. Pro-apoptotic mediators, produced by hantavirus-infected cells or nearby immune cells, cannot kill hantavirus-infected cells. They can continue to stimulate the immune cells to make mediators by which non-infected other cells can be killed. Further work is needed to link the HTNV N protein and molecules in the p53 degradation pathway. It also would be of particular interest to determine sequences in the N protein that influence p53 degradation through the expression of deleted mutants of the N protein as in the study by Ontiveros et al. (2010). Finally, the involvement of other N protein-interacting proteins, especially HIPK2, SUMO-1, and ubc9, also needs research.

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