Am J Transl Res 2009;1(4):367-380
www.ajtr.org/AJTR906003

Original Article

HSP70 induced by Hantavirus infection interacts with viral nucleocapsid protein and its overexpression suppresses virus infection in Vero E6 cells

Lu Yu, Ling Ye, Rong Zhao, Yan Fang Liu, Shou Jing Yang

Department of Pathology, Xi Jing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032, China

Received June 18, 2009; accepted July 8, 2009; available online July 15, 2009

Abstract: Hantavirus (HTV) infection is known to induce innate cellular response, a more specified cellular response in the host cells. However, whether it stimulates synthesis of stress proteins, particularly associations of viral proteins, is entirely unknown. The primary focus of this research is using Vero E6 cells infected with Hantaan 76-118 (HTNV) as an in vitro infection model to examine the individual contribution of HTV infection to heat shock response. This study shows that HTNV infection rapidly induced HSP70 expression in Vero E6 cells, which underwent a nucleo-cytoplasmic shuttle that lasted for more than 3 d. The increased HSP70 was preceded by induction of HSP70 mRNA. The physical association of HSP70 with viral nucleocapsid protein (NP) in infected cells was demonstrated by co-localization and immunoprecipation. Vero E6 cells that constitutively overexpress HSP70 after stable transfection with HSP70 gene, when infected with HTNV, showed selectively reduced NP synthesis. These findings suggest HSP70 is actively involved in the control of the expression level of viral structural proteins and possibly involved in virus assembly by binding of NP to HSP70. Overexpression of HSP70 does not favor viral propagation.

Key words: Hantavirus infection, Nucleocapsid protein, virus replication, HSP70, heat-shock proteins, transcription, gene transfection

Introduction

Hantaviruses (HTVs) are rodent-borne members of the family Bunyaviridae. They possess a single-stranded, negative-sense RNA genome contained within three separate segments referred to as the small (S), medium (M), and large (L) segments, encoding for the nucleocapsid protein (NP), glycoprotein precursor of two viral glycoproteins (G1 and G2), and viral RNA polymerase, respectively. To date, four distinct pathogenic serotypes of HTVs, including Hantaan virus (HTNV), Seoul virus (SEOV), Puumala virus (PUUV), and Dobrava virus (DOBV), have been identified to associate with hemorrhagic fever with renal syndrome (HFRS) in humans [1]. The pathogenesis of HTV infections is not fully understood. In humans, HTVs infect primarily endothelial cells in HFRS, but do not have an apparent direct cytopathic effect on these cells [2]. This fact suggests it is thus unlikely that direct viral cytotoxicity is the plausible mechanism for pathogenicity in vivo.

Host cell responses to metabolic or environmental stresses are generally mediated by induced synthesis of a selected family of proteins, collectively known as stress proteins or heat-shock proteins (HSPs) [3]. HSPs are highly conserved and ubiquitous molecules with an essential defense mechanism for protecting cells from various environmental damages [4]. HSPs mainly function as molecular chaperons and are involved in many biological processes [5]. The HSP70 family is the most abundant in the cells and well studied among others [6]. Undergoing a nucleo-cytoplasmic shuttling and subsequent nuclear accumulation of HSP70 is an early

functional event upon exposure to stresses [7].

Infections by several other viruses can induce cellular stress responses, which are characterized by expression of stress response proteins, such as HSPs [8]. HSP induction has been found in a number of different cells following infection by a variety of RNA and DNA viruses [8-10]. The transient association of HPSs with viral proteins and the presence of HPSs in virions have been described [11]. These indicate that HSPs play roles in the carrying out of the viral life cycle in host cells. It has been demonstrated that induction of a large number of HSPs, most notably HSP70, gives rise to antiviral activity during various viral infections [12], and in other instances, stimulates viral entry, transcription, nuclear translocation, and virion formation [13].

HTV infection in vitro is known to induces innate responses, a more specified cellular response in host cells [14]. Among them are expression of interferons (IFNs) and IFN-inducible genes, which show antiviral activity against HTVs [15]. Such response to HTV infection seems to be varied among host cell species and the types of pathogenic and nonpathogenic HTVs. A striking difference in the early induction of host IFN-stimulated genes by pathogenic and nonpathogenic HTVs has been observed in several endothelial cells with potential roles in pathogenesis [14-16]. However, little is known about the specific cellular stress responses to HTV infections.

In this study, HTV- permissive Vero E6 cells are used as an in vivo infection model to address whether cellular HSP70 is induced during HTV infection, and whether HSP70 is associated with HTV proteins within the cells. These findings show that HSP70 mRNA and protein levels rapidly increase in response to HTNV infection in parallel with viral gene products in Vero E6 cells. A substantial fraction of HSP70 was found to be associated with HTNV NP in the infected cells by immunoprecipitation. Alternatively, overexpression of HSP70 in Vero E6 cells by HSP70 gene transfection, when infected with HTNV, show significantly decreased HTNV NP levels with a correlated cellular tolerance to infection. Thus, it is postulated that HSP70 may be involved in HTV assembly, and host cell factors may elicit defensive responses that disrupt HTV viral replication.

Materials and Methods

Virus, cell culture, and antibodies

Vero E6 cell line (American Type Culture Collection, Rockville, Md.) was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y.). Hantaan virus strain 76-118 was propagated in Vero E6 cells. Antibodies used include rabbit anti-HSP70 polyclonal antibody (PAb) (A500; DAKO,

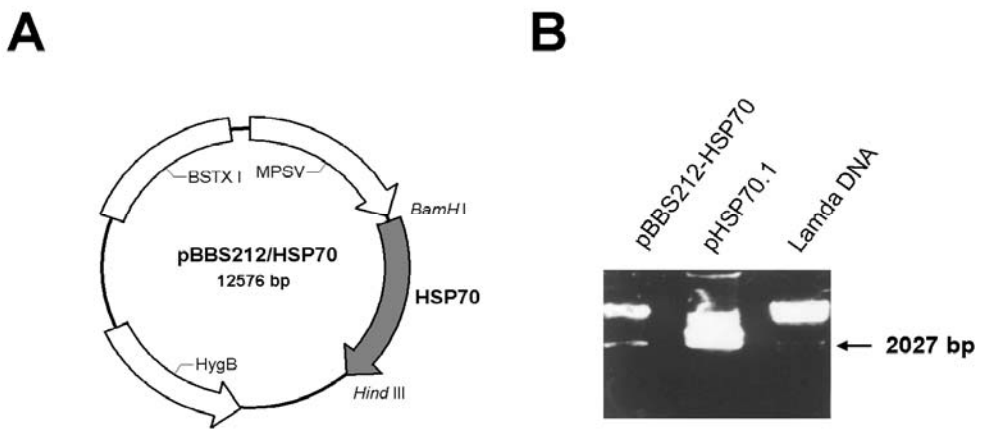

Figure 1. Construction of human HSP70 gene expression plasmid. (A) Schematic representation of plasmid encoding human HSP70. A DNA fragment containing the complete HSP70 gene was PCR-amplified from plasmid pHSP70.1, and cloned at the *BamH* I and *Hind III* sites of pBBS212, generating pBBS212-HSP70. The locations of the hygromycin B resistance gene (hyg), Cat gene (cat), and the multiple restriction sites are indicated. (B) Analysis of constructs by restriction enzymatic digestion.

Hamburg, Germany), mouse anti-human HSP70 monoclonal antibody (MAb) (W27; Santa Cruz Biotechnology, Santa Cruz, CA) and HTNV NP-specific MAb, clone 1A8, as described previously [17].

Probes and Labeling

For dot blot hybridization, an oligonucleotide probe for HSP70 (5'-GCTAAGCAGTTGGTGGTG CAGGA-3') was raidoactively labeled on 5' end by T4 polynucleotide kinase with [γ-^{32}P] ATP (Amersham Pharmasia Biotech, Bucking - hamshire, UK) (5×10^6 cpm/ml) subject to the manufacturer's specifications. For in situ hybridization, plasmid pHSP70.1 [18], which contained HSP70 cDNA fragment and plasmid pGEM1-HTNV S [19], which contained Hantaan virus S fragment DNA, were digested with restriction enzymes, respectively. The linerlized plasmids were used as templates to generate digoxigenin-labeled cDNA probes using a digoxigenin-11-dUTP random primed DNA labeling kit (Boehringer Mannheim, Germany).

Construction of human HSP70 expression vector and transfection of Vero E6 cells

The mammalian expression plasmid, pBBS-212-HSP70, shown in **Figure 1A**, was constructed for transfection of Vero E6 cells in vitro. Plasmid pBSS212, previously described as pMPSV-TM [20], contains a MPSV promoter, the CMV enhancer, and the SV40 polyadenylation site. A human HSP70 DNA fragment was obtained from plasmid pHSP70.1 by digestion with *BamH* I and *Hind* III, and cloned into *BamH* I and *Hind* III sites of pBBS212 by ligation with T4 DNA ligase, generating pBBS212-HSP70, which contains a hygromycin resistance gene. The construct was digested with *BamH* I, and *Hind* III (**Figure 1B**), and further sequenced to verify the accuracy.

Transfections were performed after the cells had reached more than 70% confluency using a transfection reagent LipofectAMINE (Life Technologies) according to the manufacturer's instructions.

Virus infection

Cell monolayers grown near confluency were exposed to virus 0.5 MOI (multiplicity of infection) in a serum-free medium for 3 or 5 h. After the unabsorbed viruses were removed and the medium containing 10% FBS was added, cells were incubated at 37°C. At 0.5, 2, 4, 6, 8, 24 h, and 2, 3, 5, 6, and 7 d post infection (p.i), the HTNV-infected monolayers were harvested and processed for experiments.

Immunohistochemistry

The cells were incubated with anti-HSP70 MAb diluted 1:200 or anti-NP MAb diluted 1:1,000 at 4°C overnight, followed by several washing with PBS (phosphate buffered saline). The specific binding of antibodies to antigens was detected by incubation with biotinylated rabbit anti-mouse IgG for 1h at 37°C. Immunoreactivity was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB).

Immunofluorescence and confocal microscopy

The cells on coverslips were incubated with anti-HSP70, 1:200 in PBS containing 1% bovine serum albumin (BSA) at 4 °C overnight. Subsequently, coverslips were washed with PBS containing 0.02% Triton X-100 and 1% NGS, and then incubated with FITC-conjugated goat anti-mouse IgG antibody at 1:40 diluted in PBS at RT for 1 h. For dual immune-fluorescent labeling, the cells were incubated with anti-HSP70 PAb diluted at 1:200 and anti-NP MAb diluted at 1:1,000 for 1 h at 37°C. The presence of HTNV NP was detected by a 1:40 dilution of FITC-conjugated goat anti-mouse IgG (Dako, USA). Specimens were examined by MRC 1024 laser scanning confocal microscopy (Bio-Rad, Hercules, CA).

Crossed-immunoprecipitation enzyme-linked immunosorbent assay (ELISA)

An anti-NP MAb, anti-HSP70 PAb or isotype control MAb (2 μg/well) was used as the capture antibody to coat 96-well microtiter ELISA plates. Absorbance at 492 nm was read in an automated ELISA reader (Bio-Rad, Hercules, CA).

Immunoprecipitation and immunoblotting

Cell lysates were prepared from mock-infected, or virus-infected cells, subsequently incubated with protein A/G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C, centrifuged, washed five times with lysis buffer, and separated on an sodium dodecyl sulfate (SDS)- polyacrylamide gel electro-phoresis (PAGE) in 10% polyacrylamide gel.

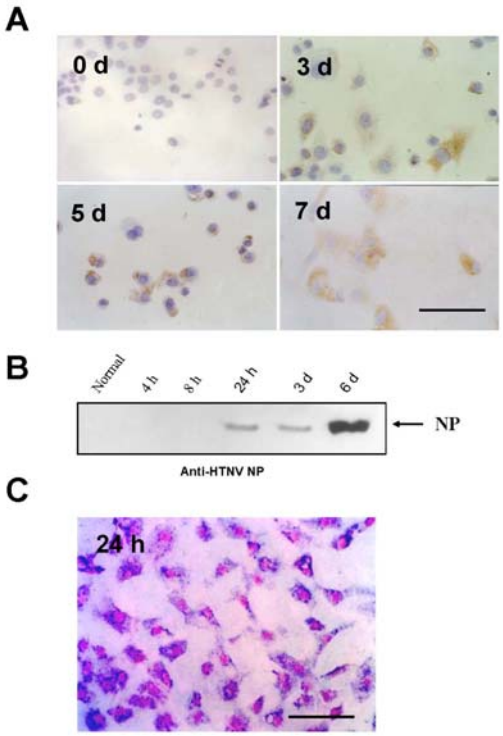

Figure 2. HTNV infection in Vero E6 cells. (A) Kinetics of viral NP immunoreactivities in HTNV-infected cells. (B) Western blot analysis of NP synthesis following HTNV infection. (C) In situ hybridization detection HTNV S segment RNA in infected cells. The HTNV infected Vero E6 cells showed strong blue hybridization signals in the cytoplasm. Scale bars, 50 μm.

The SDS-PAGE was then subjected to immunoblot analyses using anti-NP MAb (1:2,000) or anti-HSP70 PAb (1:200).

Western blot analysis

Cell cultures were harvested at designated time intervals and counted. An equal amount of total cell lysates were separated by SDS-PAGE and probed for HSP70 using 1:200 dilution of a mouse anti-HSP70 MAb, or for HTNV using 1:2,000 dilution of anti-NP MAb.

Semiquantitative reverse transcription (RT) - polymerase chain reaction (PCR)

Total cellular RNA was extracted with TRIzol reagent (Invitrogen, San Diego, Calif.). The resulting cDNAs were used as templates for PCR amplification with the primers HSP70 forward (5′-AAGGTGGAGATCATCGCCAA-3′) and HSP70 reverse (5′-GCGATCTCCTTCTTCATCTT GGT-3′). As a control for the input RNA, levels of GAPDH (forward primer, 5′-AATTCAACGGCA CAGTCAAGGC-3′; reverse primer, 5′-GGATGCA GGGATGATGTTCTGG-3′) were assayed. The PCR products were analyzed by electro-phoresis on a 1.5% agarose gel.

Dot blot hybridization

Dot blot hybridization was carried out to determine HSP70 mRNA levels. Total RNA was isolated from 5 to 10 $\times 10^6$ cells using the guanidinium isothiocianate method. The ratio between the signal intensities for HSP70 and GAPDH mRNA was calculated for the 7.5, 15, and 30μg RNA dots, and the mean of these calculations was used for each experiment.

In situ hybridization

In situ hybridization was performed using a DIG DNA labeling and detection kit (Boehringer Mannheim, Germany) and cells were counterstained with nuclear Fast Red (Sigma Chemical Co., St. Louis, MO). A 550-bp fragment of the pBR322 cDNA was used as a positive control for hybridization.

Statistical analysis

All values are expressed as means ± SEM. One-tailed Student's *t*-test was used to determine significant differences between experimental and control groups. A *P* value of 0.05 or less was considered significant.

Results

HTNV infection in Vero E6 cells

Immunocytochemical analysis of intracellular viral NP was used to determine the proportion of cells that were infected following viral exposure. The kinetics of viral infection in Vero E6 cells are shown in **Figure 2A**. HTNV NP was first observed at 2 d p.i, and largely confined to the cytoplasm of virus-infected cells during the detection of HSP70 induction. Significant viral NP was present in more than 95% of acutely infected cells by more than 3 d. Staining was not observed in any of the uninfected HTNV NP cells.

Western blots were utilized to determine levels of HTNV NP at different times following viral

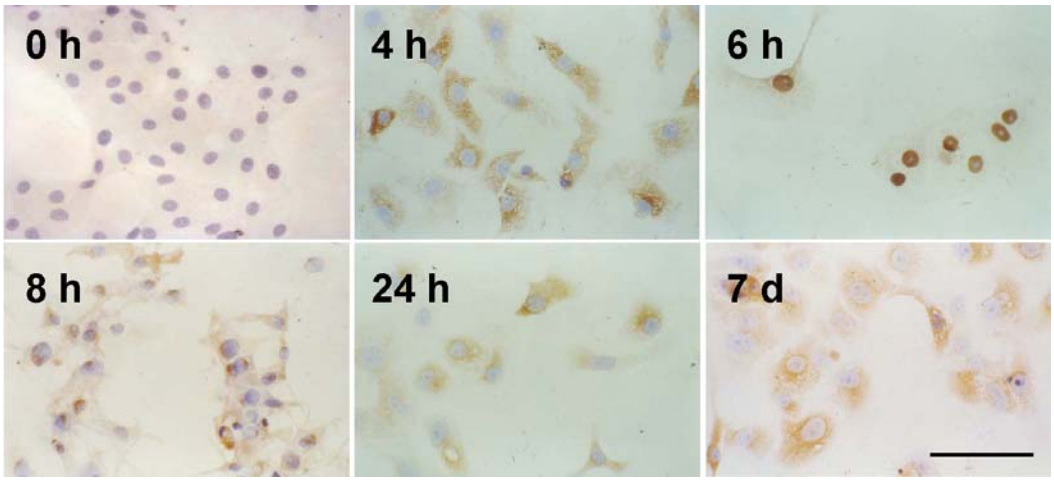

Figure 3. HSP70 induction and localization in HTNV-infected Vero E6 cells. HSP70 presented in the cytoplasm at 4 h p.i, translocated to nucleus at 6 h p.i, returned to cytoplasm at 8 h p.i, and remained at high levels at 24 h p.i, and 7 d p.i. Scale bars, 50 μm.

infection. As shown in **Figure 2B**, a single protein band of 43 kD was detected in proteins extracted from Vero E6 cells as early as 24 h following HTNV infection. The NP level was not apparent until 6 d after infection.

The cells were also treated for in situ hybridization with viral S RNA strand-specific probes. Viral RNA was first observed in the cytoplasm of infected cells as early as 24 h p.i. Infection was widespread and almost all the cells were infected (**Figure 2C**). The non-specific pBR322 cDNA did not hybridize HTNV-infected cells, nor uninfected cell cultures.

Thus, the former results show that we have developed an effective system for monitoring stress protein expression during a relatively synchronous infection of all Vero E6 cells.

HSP70 protein induction in Vero E6 cells following HTNV infection

Immunocytochemistry was used to show the dynamic subcellular localization of HSP70 during HTNV infection (**Figure 3**). HSP70 expression first appeared 4 h after HTNV infection. The cytoplasmic localization of HSP70 and continuous accumulation of HTNV NP were observed in a number of HTNV-infected cells. At 6 h p.i, the most intense nuclear staining for HSP70 was observed. At 8 h p.i, HSP70 nuclear staining decreased in intensity while cytoplasmic staining was dominant, even though nuclear staining for HSP70 was still observed in a few HTNV-infected cells during this period. At 24 h p.i, all cells were positive for HSP70 induction and remained in the cytoplasm throughout the experiment.

After viral infection, the relative intensities of HSP70 immunofluorescence in Vero E6 cells were measured. As shown in **Figure 4**, HTNV infection leads to increased expression of HSP70 in Vero E6 cell cultures with HSP70 localized mainly in the cytoplasm in infected cultures when compared with mock-infected cultures ($P < 0.05$) (**Figure 4B**). This distribution and increased expression of HSP70 was peaked at 8-24 h p.i. By contrast, the increase in expression and nuclear translocation of HSP70 were not observed in mock-infected cultures, indicating the basic level of HSP70, which was in accordance with the data from other experiments described here.

To further determine whether HTNV infection resulted in HSP70 induction, total cellular extracts from control and infected cells at various times were subjected to Western blot analysis. As depicted in **Figure 4C**, a single protein band of 70 kD was detected in proteins extracted from Vero E6 cells after incubation with the HSP70-specific antibody. This specific band showed intensity 4 h p.i, suggesting that HSP70 is highly expressed after viral infection.

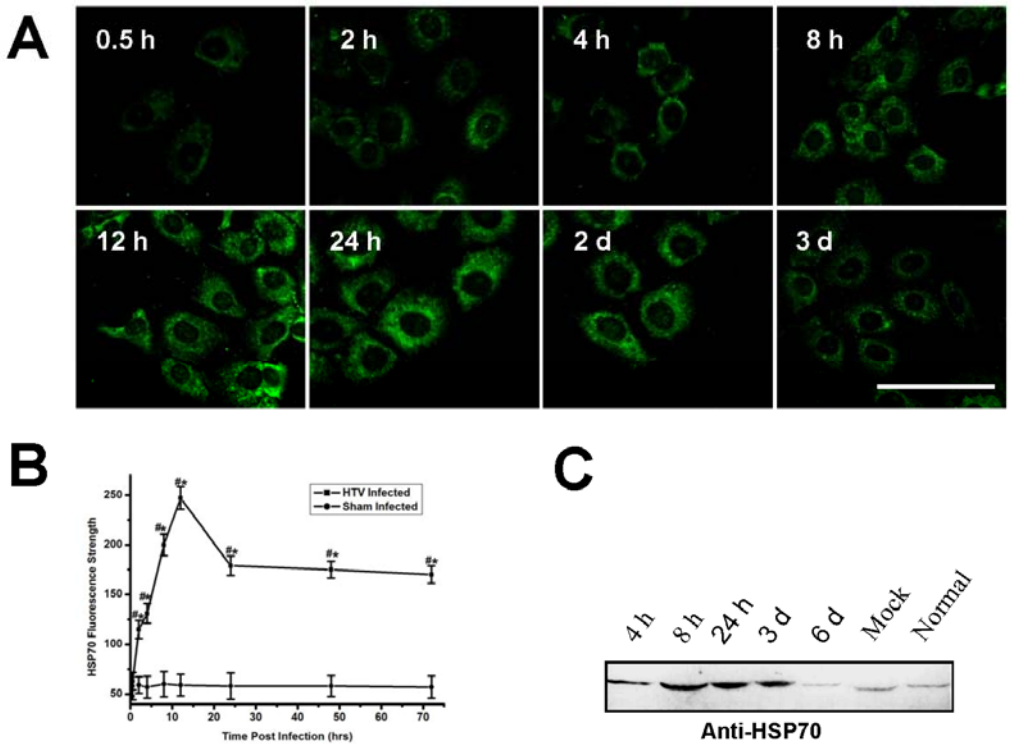

Figure 4. HTNV infection induced HSP70 protein expression in Vero E6 cells. (A) Kinetics of HSP70 immunoreactivities in HTNV- infected cells. HSP70 was present in the cytoplasm in Vero E6 cells 0.5, 2, 4, 8, 12, and 24 h, 2 and 3 d p.i. Scale bars, 50 μm. (B) Depicts relative intensity measurement of HSP70 immunofluorescence in infected cells. A significant change in intensity was observed between mock-infected controls and HTNV infected cultures. *, $p < 0.05$ vs 0 h; #, $p < 0.05$ vs mock infection. (C) The time course of HSP70 induction in Vero E6 cells after infection with HTNV was indicated by Western blot analysis. Controls are Vero E6 cells without treatment.

Maximal induction of HSP70 protein synthesis occurred between 8 and 24 h following infection prior to viral NP synthesis, as indicated by the absence of viral NP. In all of these experiments, peak HSP70 inductions were gradually down-regulated after 3 d of infection, in association with the initiation of viral protein synthesis, and then returning to baseline levels at 6 d p.i. This upregulation of HSP70 expression was detected only in infected cells, whereas no such response was observed in mock-infected cells. Quantitative analysis revealed a significant increase in the expression of HSP70 up to3 d with an approximate 12.19 fold increases in HSP70 peak signals in infected cell cultures as compared to others ($P < 0.05$).

HSP70 mRNA expression in response to Hantavirus infection

In situ hybridization was used to demonstrate HSP70 transcription in HTNV-infected and mock-infected Vero E6 cells, where the presence of HSP70 mRNA is visualized as a blue precipitate while cell nuclei are counterstained red. Cells visualized in the mock-infected group only showed weak blue granules, representative of HSP70 transcripts. When cells were infected with HTNV and probed for HSP70 mRNA, the hybridization signal observed was more robust, particularly in the cytoplasm (**Figure 5A**), indicating a marked increase in transcription.

Dot blot analysis was performed on the total RNA obtained from infected and control cells to evaluate the levels of HSP70 mRNA during viral infection. In **Figure 5B** an example of experimental data is shown for HSP70 mRNA from Vero E6 cells at different intervals after HTNV infection. Transcriptional modulation of

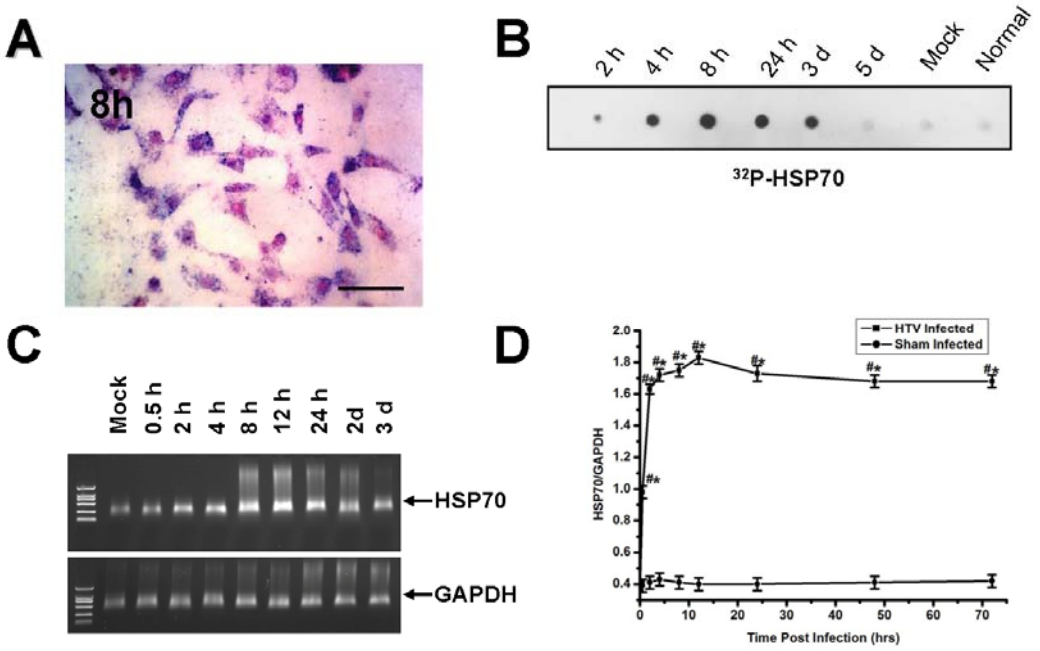

Figure 5. HTNV infection induces HSP70 mRNA expression in Vero E6 cells. (A) The infected Vero E6 cells showed strong blue hybridization signals in the cytoplasm. Scale bars, 50 μm. (B) Representative dot blots from cells showed HSP70 mRNA in cells infected by virus for 2, 4, 8, 24 h, 3 and 5 d p.i. (C) RT-PCR of the HSP70 mRNA during HTNV. Cells were infected with HTNV and harvested from 0.5 h to 3 d p.i. (D) Quantization of HSP70 mRNA by densitometry. Results are means ± SD for each group. *, $p < 0.05$ vs. 0 h; #, $p < 0.05$ vs. mock infection.

HSP70 occurred by 2 h following infection, increased by 4 h, attaining peak levels by 8 h with a 19.15 fold, higher than basal level, increase prior to the first appearance of genomic HTNV viral transcripts that appeared 24 h following infection. Substantial HSP70 mRNA transcription maintained for 3 d following infection, and gradually declined to steady state levels by 5 d in accordance with maximal levels of HTNV RNA expression. These findings indicate that acute HTNV infection leads to the selective modulation of HSP70 mRNA transcription. In contrast, HSP70 mRNA was hardly detectable in uninfected Vero E6 cells, which is consistent with other data [21, 22].

The representational experiments of RT-PCR displayed in **Figure 5C** shows the time course of HSP70 induction in response to infection. Increased HSP70 mRNA expression occurred as early as 0.5 h after viral infection and reached maximum levels between 8 h and 3 d. Thereafter, as viral RNA replication increased, the upregulation of HSP70 gene expression was declined, yet maintained at a relatively higher level than seen in the control cells. In the absence of viral infection, HSP70 mRNA, like HSP70, was hardly detectable. A semiquantitative analysis with computed densitometry showed a 4 fold greater increase in HSP70 mRNA in infected cells as compared to the uninfected cells (**Figure 5D**). Thus, the effects of HTNV infection on the levels of HSP70 mRNA parallel the effects of infection on the levels of its protein in Vero E6 cells, suggesting that the induction of HSP70 by viral infection is at least, in part, regulated at the transcriptional level.

To ensure that the induction of HSP70 mRNA was virally-associated and not due to cellular stress, a mock infection was carried out in parallel using the same viral stock with the complete removal of any viral debris by ultracentrifugation. Whereas compared to the original virus stock induced HSP70 mRNA transcription, the parallel mock infection using the ultracentrifuged viral stock failed to yield HSP70 mRNA induction. These findings

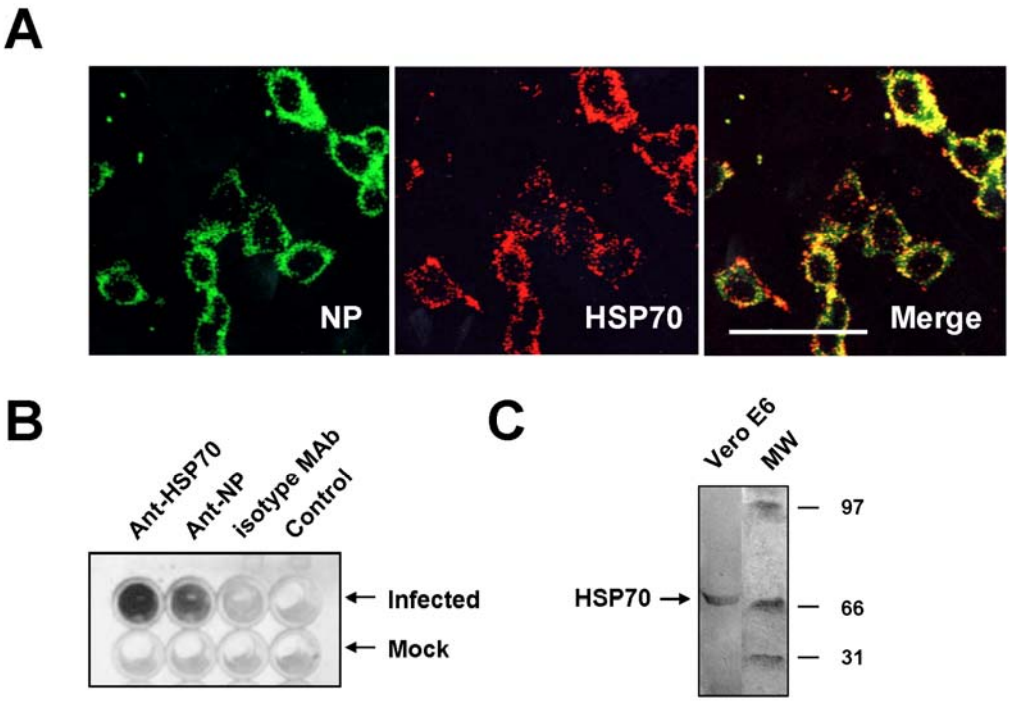

Figure 6. Analysis for association of HSP70 with NP in HTNV infected Vero E6 cells. (A) Vero E6 fixed and permeabilized for double-labeled co-localization. Strong HSP70 staining seen throughout the field co-localized with NP, shown in yellow where merged TXR/FITC images. Scale bars, 50 μm. (B) Crossed-immunoprecipitation ELISA. HSP70 was found in association with NP only in HTNV-infected Vero E6 cells. (C) Immunoprecipitation analysis of NP and HSP70 complexes in virus infected cells.

indicate that HTNV infection leads to the selective modulation of HSP70 transcription.

There were no significant differences in cell proliferation or cell viabilities between infected and mock-infected cells until 6 d p.i. The culmination of viral infection was associated with substantial increases in viral protein and viral RNA synthesis, a dramatic decline in Vero E6 cell survival, and a corresponding disappearance of a HSP70 stress response (data not shown).

HSP 70 interacts with HTNV NP in infected Vero E6 cells

Confocal microscopy was used to determine cellular distribution and co-localization of viral NP synthesis with induced HSP70 during viral infection at indicated times following infection. In virus-infected cells, the cytoplasmic staining of HSP70 was observed at 12 h p.i. These strongly stained regions became partially overlapped with intracellular NP near the nuclear periphery. At 24 h p.i, HSP70-expressing cells were overlying the infected cells, which harbored large quantities of intracellular NP that spread throughout the cytoplasmic area. This co-localization of HSP70 and NP was quite obvious at 24 h to 3 d in Vero E6 cells after infection (**Figure 6A**).

A co-localization between HSP70 and the NP within the cytoplasm strongly suggested that an interaction had taken place between the NP and HSP70. To examine this possibility, cellular extracts were prepared from mock- and virus-infected cells at 0 and 2 d p.i. The presence of HSP70-NP complexes in cellular extracts were subsequently determined by crossed-immunoprecipitation ELISA with an anti-NP specific antibody as capture antibodies, and detected by anti-HSP70 specific antibody. As shown in **Figure 6B**, HSP70 was found in association with NP only in HTNV-infected Vero E6 cells.

The possibility that HSP70 binds to NP

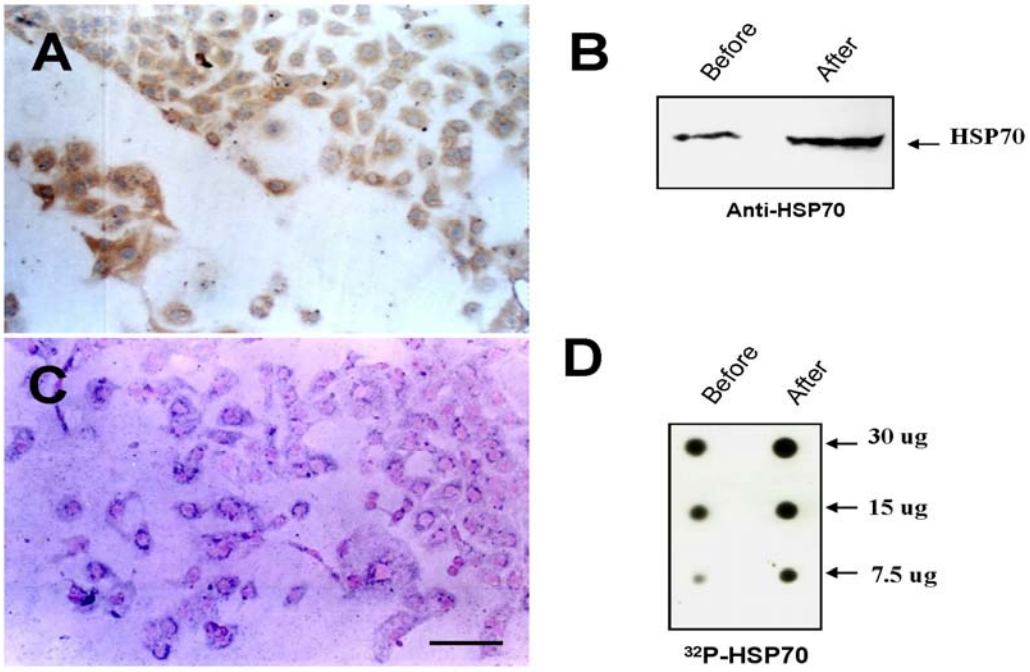

Figure 7. HSP70 mRNA and protein over-expression in Vero E6 cells transfected with HSP70 gene. (A) Immunohistochemical analysis of HSP70 expression in HSP70 gene transferred Vero E6 cells. (B) Western blotting of HSP70 expression. (C) In situ hybridization detection of HSP70 mRNA in transfected cells. Scale bars, 50 μm. (D) Dot blot of HSP70 mRNA. HSP70 mRNA levels in the Vero E6 cells before and after HSP70 gene transfection. The ratio between the signal intensities for HSP70 and GAPDH mRNA was calculated for the 30, 15, and 7.5 μg RNA dots.

transiently in infected Vero E6 cells was further examined via immunoprecipitation under the same conditions. The results showed a significant level of HSP70 in samples, which were immunoprecipitated from virus-infected cells but not from mock-infected cells (**Figure 6C**). Again the physical association of HSP70 with NP was readily detectable in infected Vero E6 cells.

The effect of HSP70 gene transfection on HTNV infection in Vero E6 cells

To evaluate transfection efficiency, the selected transfected cells were submitted to detection of HSP70 mRNA using ^{32}P- or digoxigenin-labeled probes specific for HSP70. As shown in **Figure 7**, HSP70 gene transfection introduced a maximal 5.86 fold increase in HSP70 mRNA expression from cultures derived from transfected cells as compared to those from non-transfected cells (**Figure 7D**). By in situ hybridization, transfected cells had strong HSP70 mRNA signals in their cytoplasm (**Figure 7C**), whereas the non-transfected cells showed a low yet detectable level. An immunohistochemical examination and Western blot with anti-HSP70 MAb showed an apparent maximal 5 fold increase in HSP70 overexpression in the cytoplasm of transfected Vero E6 cells (**Figure 7A** and **7B**) as compared with those from the non-transfected cells (**Figure 3**). The transfected cells showed a distinctively stronger staining than the control cells indicating that the transfected Vero E6 cells were overexpressing HSP70. These findings suggest transfected cells can stably express HSP70 mRNA and proteins.

To investigate the effect of HSP70 over-expression on viral infection and HTNV NP synthesis, HSP70 gene transfected and non-transfected Vero E6 cells were subjected to HTNV infection and then analyzed for HTNV NP and HSP70, respectively. In non-transfected Vero E6 cells, high levels of inducible HSP70 expression was observed following HTNV infection. Strong staining intensities for HSP70 were seen throughout the cytoplasm with the majority of cells. HTNV infection was also

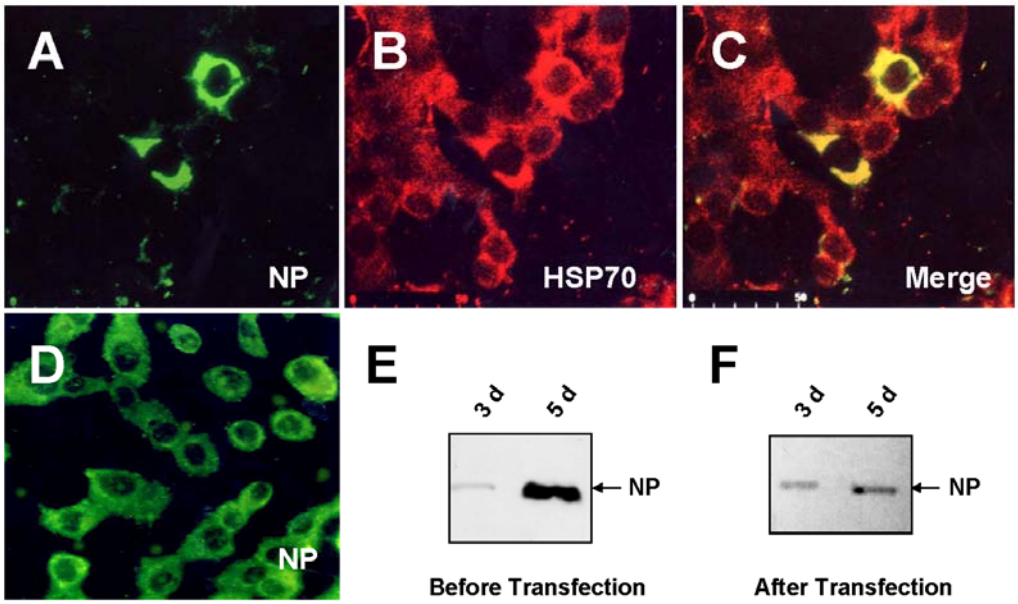

Figure 8. HSP70 gene transfection reduces HTNV infection in Vero E6 cells. (A, B, C) Only few Vero E6 cells showed positive for HTNV NP as detected by FITC, resulting in only few cells co-localized with HSP70. (D) Non-transfected Vero E6 cells highly expressed HTNV NP after virus infection. Scale bars, 50 μm. (E, F) Western blot analysis of HTNV NP synthesis. HTNV NP was checked by Western blot with anti-HTNV NP MAb in non-transfected (E) or HSP70 transfected Vero E6 cells (F) 3 and 5 d after virus infection. Apparent reduced NP synthesis was observed only in cells transfected with HSP70 gene.

observed throughout the cell slides with virtually every cell positively stained for NP by anti-NP MAb. Co-localization showed that the HSP70-expressing cells were overlying the infected cells, producing yellow, indicative of a response to the onset of virus replication (**Figure 6**). No co-localization was found with control uninfected cells. In HSP70-transfected cells, there was a marked cell population of increased HSP70 accumulation in the cytoplasm, which lay close to the front of HSP70 expression, as revealed by probing for HSP70 mRNA (**Figure 7D**). In contrast, only a few cells were found to express NP within the populations of HSP70-expressing cells (**Figure 8A, 8B** and **8C**), which colocalized with HSP70, when compared with non-transfected cell (**Figure 8D**). This result showed that the upregulation of HSP70 gene expression was followed by inhibiting the viral infection and a decline in viral protein synthesis after infection.

HTNV NP production in HSP70 gene transfected Vero E6 cells following HTNV infection was further analyzed by Western blot. In this case, the levels of intracellular NP were dramatically reduced by approximately 15.15 fold at 5 d p.i. in HSP70 gene transfected Vero E6 cells (**Figure 8F**), when compared with non-transfected Vero E6 cells (**Figure 8E**).

Discussion

This study demonstrates for the first time that HTNV infection induces a stronger stress response in Vero E6 cells, which is characterized by rapid and transient increases of HSP70 mRNA transcription and protein expression upon HTNV infection, resulting in a physical association of HSP70 with HTNV proteins. Inversely, overexpression of HSP70 by HSP70 gene transfection in Vero E6 cells reduces viral NP production following HTNV infection and increase cell viability. Thus, this evidence shows that host HSP70 stress pathways are selectively implicated in HTNV infection.

Hantavirus infection induces HSP70 expression in Vero E6 Cells

HTNV infection induces a rapid upregulation of HSP70 in Vero E6 cells, transiently directs

HSP70 to the nucleus in the early phase of infection but redirects it to the cytoplasm in the late phase, coincided with the onset of viral replication, indicating HTNV infection induced heat shock response. Hence, many of the features of the early response to HTV replication mimic the general cellular stress during other viral infection [23, 24]. Viral induction of the major HSP 70 expression and nucleo-cytoplasmic shuttle may simply be a part of the generalized stress response to viral infection or an early event mainly at the protein level [23, 25], and play no role in viral replication [21, 26]. Thus, the rapid and transient induction of HSP70 synthesis at early time of infection is the consequence of HTNV entry into Vero E6 cells and likely reflects the generation of an adaptive response of the host cell to resist the viral attack. However, after the transient nucleo-cytoplasmic shuttle, a long-lasting HSP70 mRNA and protein expression is followed. Cumulative findings indicate that the induction of HSP70 is not a general response to viral infection but, instead, a highly specific response with regard to both the infecting virus and the host cell [25], even though no clear consensus exists so far to explain the likely reasons for HSP70 induction within host cells during viral infection. This definitively demonstrates that up-regulation of the chaperone machinery is not simply an indirect consequence of the stress induced by infection, but most likely is required for viral functions [27]. This suggests that stress proteins are likely involved in different steps in the virus replication cycle.

HSP70 associates with Hantavirus NP in Vero E6 cells

The facts that HSP70 induced by HTNV infection co-localized and interacted with NP within cytoplasm, and a significant level of HSP70 immunoprecipitated with NP from virus-infected cells, strongly suggest HTNV proteins physically associate with HSP70 during viral infection. The transient association of HSP with viral proteins and assembly intermediates during virus replication has been described in many other virus systems [21, 28].

As a rule, viruses do not encode HSP70s, and hence, HSP expression in the cells infected with these viruses represents cellular origin. It is well established that HSP70 exerts a chaperone function that drives newly synthesized proteins either to a stabilized conformation or to the cellular degradation machineries. Thus, the existence of chaperone interaction may be a consequence of the recruitment of HSP70 to sites of viral protein expression [10], perhaps involved in an intermediary role in interfering with viral protein synthesis and replication [12, 29], suggesting that cellular HSPs play an important role during viral replication [30]. The possibility that HSP70 could be beneficial for the host has been suggested [31].

HTV NP, a major structural component of a virion, is found to be a multifunctional viral macromolecule interfering with important regulatory pathways in the infected cells [32]. HTV NP can form stable trimers and interact with viral RNA. They are thought to serve as intermediates in the process of oligomerization and ribonucleoprotein formation [33]. Besides this structural function, HTV NP may also have some ambassador functions, interacting with cellular machinery, such as small ubiquitin-like modifier-1, small ubiquitin-like modifier-1–interacting proteins and Fas-mediated apoptosis enhancer Daxx [34], suggesting a direct link between host cell machinery and a hantavirus structural component. The finding that HSP70 is induced in abundance in virus-infected cells, and transiently associates with NP, it may act as a potential cofactor for HTNV replication, adding to the information about the interactions between HTNV NP and host cellular proteins.

HSP70 overexpression reduces NP synthesis in Vero E6 Cells

To clarify the effect of cellular HSP70 on HTNV infection in Vero E6 cells, the HTNV NP level was examined under conditions where the intracellular HSP70 level was manipulated. HSP70 gene transfection of Vero E6 cells initiated, as expected, a strong and prolonged HSP70 expression as compared to innate inducible HSP70. If increased cytoplasmic concentrations of HSP70 promote formation of HSP70-NP complexes, then HSP70-mediated increases in HSP70 overexpression may reflect a decreased amount of free nucleocapsid protein available for virion assembly. Indeed, these stably transfected clones, when infected with HTNV, showed significantly decreased HTNV NP levels 5 d after viral infection with a correlated cellular tolerance to infection as compared with the

control or untreated cells. This is consistent with what can be expected from the above conclusion. These findings appear to provide the evidence that overexpressed HSP70 plays a direct role in preventing the virus protein synthesis in target cells and in the enhancement of cellular tolerance in vitro.

The possibility that HSP70 could be a mediator of antiviral effect is supported by the fact that treatment with other classical inducers of HSP70, including cyclopentenone prostaglandins, sodium arsenite, cadmium, and heat shock, selectively prevented virus protein synthesis [35]. It is hypothesized that the antiviral effects by induced HSP70 is possibly through a chaperon function of HSP70 interfering with viral protein synthesis and replication [12, 29]. In the case of canine distemper virus, a low level of HSP72 may actually be optimal for promoting viral gene expression whereas higher levels inhibit transcription [36]. Our study indicates that overexpression of HSP70 within the virus-infected cell did detectably alter viral replication. Although the nature of the interaction between the NP and HSP70 remains to be characterized, the association of NP with HSP70, during viral infection, may be interpreted to reflect normal chaperone events, such that a plentiful supply of HSP70 with inherent affinity for nonnative protein would be highly likely to interact with abundant nascent viral proteins present in the same cellular compartment. This might negatively regulate NP, such as its transcription factor-like activity [32], or control the amount of viral protein available for virus morphogenesis. Thus, confirming the observation made by reduced NP synthesis.

Overexpression of HSP70 has been shown to improve cell tolerance to inflammatory cytokines, such as TNF-α and IL-1 [37, 38], and protect cells from stress-induced apoptosis [39]. These facts are well in agreement with the finding that Vero E6 cells with high expression levels of HSP70 by HSP70 gene transfection shows enhanced tolerance to HTNV infection being presented in this study. Therefore, the present model with gene transfection might be more suitable for investigating the effect of HSP70 on viral infection, and appears to be more effective and advantageous when applying HSP70 to clinical treatment. For this purpose, however, further investigation and improvement remain necessary.

Implications for immune response to infection

HSPs may act as molecular links bridging innate and adaptive immune responses by participating in antigen presentation and eliminating virus-infected cells [40]. HSP70 induced by HTNV infection, as described here, may have implications for immune recognition of virus-infected cells. The possible mechanism of cytoplasmic HSP70 action is its involvement in the virus-induced transport of major histocompatibility complex class I molecules from the endoplasmic reticulum to the cytosol [41], and facilitates viral antigen presentation in cells such as macrophages and dendrites [40]. The pathogen-derived stress proteins are frequent targets of T cells during the immune response to infection [42]. Moreover, these stress proteins, during HTV infection, accumulate to substantial levels, especially complexed with HTNV NP, if released from necrotic infected cells into the serum and appropriately presented. These complexes can elicit cytotoxic T lymphocyte (CTL) responses against peptides bound to HSP70 [43] because functional CTLs are required for clearance for viral infection [44]. This capability would provide a first line of defense against infection by permitting recognition and elimination of virus infected cells, and in other instances, bring about immunopathogentic lesion. Actually, HSP70-NP complexes do possess the ability to enhance NP specific CTL responses [45, 46].

In summary, present study shows that HSP70 expression is rapidly induced after HTNV infection, resulting in a substantial proportion of HSP70 association with HTNV NP for more than 3 d after viral infection, and down-regulated at a later stage of infection. Conversely, overexpression of HSP70 by gene transfection results in the inhibition of viral NP synthesis. These results demonstrate an inverse relationship between HSP70 expression and viral protein synthesis, suggesting that HSP70 is important for the HTNV life cycle, and its induction by HTNV may play a role in the pathogenesis of HFRS and its sequelae.

Acknowledgments

The authors would like to thank Dr. Zhi Kai, Xu (Department of Microbiology, 4th Military

Medical University, Xi'an, 710032, China) for kindly providing the 1A8 MAb. The authors would also like to thank Dr. Gavin Lawlis (Center for Cardiovascular Research and Alternative Medicine, Division of Pharmaceutical Sciences, University of Wyoming, Laramie, Wyoming 82071, USA) who provided helpful improvement for this manuscript. This work was supported by a Grant from the National Natural Science Foundation of China (No. 30271186).

Please address correspondences to: Shou Jing Yang, MD, PhD, Department of Pathology, Fourth Military Medical University, No. 17 Chang Le Xi Road, Xi'an, Shaanxi 710032, Tel: (011) 86 29-84773527, Fax: (011) 86 29-84773624, E-mail: yangsj@fmmu.edu.cn

References

[1] Schmaljohn CS, Hasty SE, Dalrymple JM, LeDuc JW, Lee HW, von Bonsdorff CH, Brummer-Korvenkontio M, Vaheri A, Tsai TF, Regnery HL and et al. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science 1985; 227: 1041-1044.

[2] Temonen M, Mustonen J, Helin H, Pasternack A, Vaheri A and Holthofer H. Cytokines, adhesion molecules, and cellular infiltration in nephropathia epidemica kidneys: an immunohistochemical study. Clin Immunol Immunopathol 1996; 78: 47-55.

[3] Morimoto RI. Heat shock: the role of transient inducible responses in cell damage, transformation, and differentiation. Cancer Cells 1991; 3: 295-301.

[4] Morimoto RI. Cells in stress: transcriptional activation of heat shock genes. Science 1993; 259: 1409-1410.

[5] Gething MJ and Sambrook J. Protein folding in the cell. Nature 1992; 355: 33-45.

[6] Morimoto RI and Milarski KL. Expression and function of vertebrate hsp70 genes. In: Morimoto RI, Tissieres A, Georgopoulos C, editors. Stress proteins in biology and medicine. New York: Cold Spring Harbor Laboratory, Cold Spring Harbor; 1990. p. 323-360.

[7] Sanger F, Nicklen S and Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 1977; 74: 5463-5467.

[8] Lindquist S and Craig EA. The heat-shock proteins. Annu Rev Genet 1988; 22: 631-677.

[9] Jindal S and Malkovsky M. Stress responses to viral infection. Trends Microbiol 1994; 2: 89-91.

[10] Mayer MP. Recruitment of Hsp70 chaperones: a crucial part of viral survival strategies. Rev Physiol Biochem Pharmacol 2005; 153: 1-46.

[11] Sagara J and Kawai A. Identification of heat shock protein 70 in the rabies virion. Virology 1992; 190: 845-848.

[12] Rozera C, Carattoli A, De Marco A, Amici C, Giorgi C and Santoro MG. Inhibition of HIV-1 replication by cyclopentenone prostaglandins in acutely infected human cells. Evidence for a transcriptional block. J Clin Invest 1996; 97: 1795-1803.

[13] Guerrero CA, Bouyssounade D, Zarate S, Isa P, Lopez T, Espinosa R, Romero P, Mendez E, Lopez S and Arias CF. Heat shock cognate protein 70 is involved in rotavirus cell entry. J Virol 2002; 76: 4096-4102.

[14] Prescott J, Ye C, Sen G and Hjelle B. Induction of innate immune response genes by Sin Nombre hantavirus does not require viral replication. J Virol 2005; 79: 15007-15015.

[15] Kraus AA, Raftery MJ, Giese T, Ulrich R, Zawatzky R, Hippenstiel S, Suttorp N, Kruger DH and Schonrich G. Differential antiviral response of endothelial cells after infection with pathogenic and nonpathogenic hantaviruses. J Virol 2004; 78: 6143-6150.

[16] Khaiboullina SF, Rizvanov AA, Otteson E, Miyazato A, Maciejewski J and St Jeor S. Regulation of cellular gene expression in endothelial cells by sin nombre and prospect hill viruses. Viral Immunol 2004; 17: 234-251.

[17] Xu Z, Wei L, Wang L, Wang H and Jiang S. The in vitro and in vivo protective activity of monoclonal antibodies directed against Hantaan virus: potential application for immunotherapy and passive immunization. Biochem Biophys Res Commun 2002; 298: 552-558.

[18] Milner CM and Campbell RD. Structure and expression of the three MHC-linked HSP70 genes. Immunogenetics 1990; 32: 242-251.

[19] Schmaljohn CS, Jennings GB, Hay J and Dalrymple JM. Coding strategy of the S genome segment of Hantaan virus. Virology 1986; 155: 633-643.

[20] Lin JH, Wang M, Andrews WH, Wydro R and Morser J. Expression efficiency of the human thrombomodulin-encoding gene in various vector and host systems. Gene 1994; 147: 287-292.

[21] Jindal S and Young RA. Vaccinia virus infection induces a stress response that leads to association of Hsp70 with viral proteins. J Virol 1992; 66: 5357-5362.

[22] Theodorakis NG and Morimoto RI. Posttranscriptional regulation of hsp70 expression in human cells: effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. Mol Cell Biol 1987; 7: 4357-4368.

[23] Furlini G, Vignoli M, Re MC, Gibellini D, Ramazzotti E, Zauli G and La Placa M. Human immunodeficiency virus type 1 interaction with the membrane of CD4+ cells induces the synthesis and nuclear translocation of 70K heat shock protein. J Gen Virol 1994; 75 (Pt

1): 193-199.
[24] Ohgitani E, Kobayashi K, Takeshita K and Imanishi J. Biphasic translocation of a 70 kDa heat shock protein in human cytomegalovirus-infected cells. J Gen Virol 1999; 80 (Pt 1): 63-68.
[25] Broquet AH, Lenoir C, Gardet A, Sapin C, Chwetzoff S, Jouniaux AM, Lopez S, Trugnan G, Bachelet M and Thomas G. Hsp70 negatively controls rotavirus protein bioavailability in caco-2 cells infected by the rotavirus RF strain. J Virol 2007; 81: 1297-1304.
[26] Collins PL and Hightower LE. Newcastle disease virus stimulates the cellular accumulation of stress (heat shock) mRNAs and proteins. J Virol 1982; 44: 703-707.
[27] Sullivan CS and Pipas JM. The virus-chaperone connection. Virology 2001; 287: 1-8.
[28] Cripe TP, Delos SE, Estes PA and Garcea RL. In vivo and in vitro association of hsc70 with polyomavirus capsid proteins. J Virol 1995; 69: 7807-7813.
[29] Conti C, De Marco A, Mastromarino P, Tomao P and Santoro MG. Antiviral effect of hyperthermic treatment in rhinovirus infection. Antimicrob Agents Chemother 1999; 43: 822-829.
[30] Mager WH and De Kruijff AJ. Stress-induced transcriptional activation. Microbiol Rev 1995; 59: 506-531.
[31] Brenner BG and Wainberg MA. Heat shock protein-based therapeutic strategies against human immunodeficiency virus type 1 infection. Infect Dis Obstet Gynecol 1999; 7: 80-90.
[32] Kaukinen P, Vaheri A and Plyusnin A. Hantavirus nucleocapsid protein: a multifunctional molecule with both housekeeping and ambassadorial duties. Arch Virol 2005; 150: 1693-1713.
[33] Mir MA and Panganiban AT. Trimeric hantavirus nucleocapsid protein binds specifically to the viral RNA panhandle. J Virol 2004; 78: 8281-8288.
[34] Lee BH, Yoshimatsu K, Maeda A, Ochiai K, Morimatsu M, Araki K, Ogino M, Morikawa S and Arikawa J. Association of the nucleocapsid protein of the Seoul and Hantaan hantaviruses with small ubiquitin-like modifier-1-related molecules. Virus Res 2003; 98: 83-91.
[35] Amici C, Giorgi C, Rossi A and Santoro MG. Selective inhibition of virus protein synthesis by prostaglandin A1: a translational block associated with HSP70 synthesis. J Virol 1994; 68: 6890-6899.
[36] Oglesbee MJ, Liu Z, Kenney H and Brooks CL. The highly inducible member of the 70 kDa family of heat shock proteins increases canine distemper virus polymerase activity. J Gen Virol 1996; 77 (Pt 9): 2125-2135.
[37] Jaattela M. Overexpression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A2. J Immunol 1993; 151: 4286-4294.
[38] Jaattela M and Wissing D. Heat-shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self-protection. J Exp Med 1993; 177: 231-236.
[39] Mosser DD, Caron AW, Bourget L, Denis-Larose C and Massie B. Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. Mol Cell Biol 1997; 17: 5317-5327.
[40] Srivastava P. Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol 2002; 2: 185-194.
[41] Wiertz EJ, Tortorella D, Bogyo M, Yu J, Mothes W, Jones TR, Rapoport TA and Ploegh HL. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature 1996; 384: 432-438.
[42] Young RA. Stress proteins and immunology. Annu Rev Immunol 1990; 8: 401-420.
[43] Moroi Y, Mayhew M, Trcka J, Hoe MH, Takechi Y, Hartl FU, Rothman JE and Houghton AN. Induction of cellular immunity by immunization with novel hybrid peptides complexed to heat shock protein 70. Proc Natl Acad Sci U S A 2000; 97: 3485-3490.
[44] Araki K, Yoshimatsu K, Lee BH, Kariwa H, Takashima I and Arikawa J. Hantavirus-specific CD8(+)-T-cell responses in newborn mice persistently infected with Hantaan virus. J Virol 2003; 77: 8408-8417.
[45] Li J, Ye ZX, Li KN, Cui JH, Li J, Cao YX, Liu YF and Yang SJ. HSP70 gene fused with Hantavirus S segment DNA significantly enhances the DNA vaccine potency against hantaviral nucleocapsid protein in vivo. Vaccine 2007; 25: 239-252.
[46] Li J, Li KN, Gao J, Cui JH, Liu YF and Yang SJ. Heat shock protein 70 fused to or complexed with Hantavirus nucleocapsid protein significantly enhances specific humoral and cellular immune responses in C57BL/6 mice. Vaccine 2008; 26: 3175-3187.