

## REDUCED EXPRESSION OF ANGIOPOIETIN-1 IN HANTAAAN VIRUS-INFECTED HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS INCREASES THEIR PERMEABILITY

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**Summary.** – Hemorrhagic fever with renal syndrome (HFRS) caused by Hantaan virus (HTNV) is characterized by vascular hemorrhage and acute renal failure. Angiopoietin-1 (Ang-1) is a glycoprotein that maintains vessel integrity and reduces endothelial permeability. We found that in HTNV-infected human umbilical vein endothelial cells (HUVECs) the levels of Ang-1 mRNA and protein were reduced on days 2 and 3 post-infection (p.i.), when endothelial permeability was increased. The HTNV-stimulated permeability was reduced by treatment of HUVECs with Ang-1. The plasma Ang-1 level was lower in HFRS patients than in healthy persons. Paired plasma samples of HFRS patients revealed markedly lower Ang-1 levels during the acute phase of HFRS as compared to the convalescent phase. These findings suggested that HTNV reduced the Ang-1 expression in endothelial cells that might play an important role in the increase of vascular permeability in HFRS.

**Keywords:** Hantaan virus; angiopoietin-1; vascular permeability; hemorrhagic fever with renal syndrome

### Introduction

Viral hemorrhagic fevers (VHFs) are caused by four widely different RNA virus families, namely *Flaviviridae*, *Arenaviridae*, *Bunyaviridae*, and *Filoviridae*. VHFs are associated with severe outbreaks in humans and are included in the category of emerging infectious diseases. Even though the common clinical features of VHFs are fever and hemorrhage, the pathogenesis of VHFs differs according to the infecting virus. Hantaviruses that belong to the family *Bunyaviridae*

cause HFRS and hantavirus pulmonary syndrome in humans (Linderholm and Elgh, 2001; Nichol *et al.*, 1993). HTNV, a type species in the genus *Hantavirus*, causes a severe form of HFRS. Macroscopic pathologic findings of HFRS include hemorrhage, congestion, infarct-like necrosis, and edema in several organs (Lee *et al.*, 1980). Microscopically, HTNV-mediated hemorrhage originates from extravasation of red blood cells from capillaries rather than by a rupture of the vasculature (Kim, 1976). HTNV is not a cytolytic virus and consequently, the HFRS is based on the increased vascular permeability and coagulation disorders (Ala-Houhala *et al.*, 2002; Nolte *et al.*, 1995; Cosgriff, 1991). Tumor necrosis factor- $\alpha$ , vascular endothelial growth factor, and cytotoxic T cell-mediated mechanisms have been suggested as a cause for the increased vascular permeability associated with hantavirus diseases (Gavrilovskaya *et al.*, 2008; Hayasaka *et al.*, 2007; Niikura *et al.*, 2004; Khaiboullina *et al.*, 2000). Additionally, the reduction of von Willebrand factor upon HTNV infection of endothelial cells has been suggested as one of the factors of hemorrhage in HFRS (Cho *et al.*,

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**Abbreviations:** Ang-1 = angiopoietin-1; HTNV = Hantaan virus; HRP = horseradish peroxidase; HFRS = hemorrhagic fever with renal syndrome; HUVECs = human umbilical vein endothelial cells; IFA = immunofluorescence assay; p.i. = post-infection; Tie-2 = tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2; VHFs = viral hemorrhagic fevers

2007). However, none of the mechanisms described above is able to explain sufficiently the capillary leakage associated with HFRS.

Ang-1 is a secreted protein expressed by endothelial and periendothelial cells (Gescher *et al.*, 2004; Ahmad *et al.*, 2001). The main functions of Ang-1 are the maintenance of vessel integrity, reduction of endothelial permeability, and induction of anti-inflammatory effects (Gamble *et al.*, 2000). Ang-1 prevents the vascular permeability induced by platelet activating factor, bradykinin, and histamine (Pizurki *et al.*, 2003). Ang-1 is a ligand for tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tie-2) receptor, which is also known as endothelial-specific receptor for tyrosine kinase. Tie-2 is expressed predominantly in vascular endothelial cells (Vikkula *et al.*, 1996). Ang-1 mediates vessel maturation and maintains vessel integrity by recruiting periendothelial cells. Moreover, Ang-1 induces a chemotactic response and results in network formation, sprouting, survival and apoptosis in endothelial cells (Kim *et al.*, 2000a,b; Thurston *et al.*, 2000, 1999; Papatropoulos *et al.*, 1999; Koblizek *et al.*, 1998; Witzembichler *et al.*, 1998). However, it does not induce cell proliferation. Ang-1 protects the adult vasculature against plasma leakage and promotes firm attachment of the endothelium. In spite of the previous findings, the influence of Ang-1 on vascular dysfunction in HFRS remains unclear.

The aim of the present study was to investigate the molecular mechanism of increased vascular permeability represented in HFRS. We measured the concentration of Ang-1 protein in plasma from HFRS patients and healthy persons and also the protein and mRNA expression levels of Ang-1 in HTNV-infected HUVEC.

## Materials and Methods

**Virus and cell culture.** The HTNV strain ROK 84-105 was propagated in Vero E6 cells (ATCC CRL-1586) for the preparation of a stock virus. Vero E6 cells were grown in DMEM supplemented with 10% FBS and antibiotics (Cambrex). Culture supernatants were harvested 7 days p.i., centrifuged, and the HTNV stocks were stored at -80°C until used. HUVECs (Modern Cell & Tissue Technologies) were allowed to grow in endothelial cell basal medium-2 (Cambrex) supplemented with 2% FBS, human fibroblast growth factor, human recombinant insulin-like growth factor, ascorbic acid, vascular endothelial growth factor, human recombinant epidermal growth factor, heparin, and hydrocortisone. Confluent HUVECs obtained 48 hrs after plating were inoculated with HTNV at a multiplicity of infection of 1 and incubated for 1 hr. Then, the inoculum was removed and fresh medium was added. HTNV-infected and non-infected HUVECs (control cells) were incubated for 1, 2, 3, and 5 days p.i.

**Immunofluorescence assay (IFA)** was performed as previously described (Cho *et al.*, 2007). Briefly, infected or non-infected cells were treated with human anti-HTNV (HFRS patient's serum) or rabbit anti-Ang-1 IgG (Abcam). Then, the cells were stained with mouse anti-human IgG-FITC (Jackson ImmunoResearch Laboratories) or with goat anti-rabbit IgG-Cy3 (Sigma-Aldrich). Stained cells were washed and incubated with nuclear stain Hoechst 33258 (1:5,000), mounted in Vector shield (Vector), and cover-slipped. The slides were examined under a fluorescence microscope (Zeiss).

**Isolation of total RNA and RT-PCR.** Total RNA was isolated using the RNA-Bee™ system (Tel-Test). RNA yields were quantified by A260 measurements. RT-PCR was performed as previously described (Kim *et al.*, 2007). The primer sequences were for glyceraldehydyl-3-phosphate-dehydrogenase (GAPDH) forward 5'-ACC CAC TCC ACC TTT G, reverse 5'-ATC TTG TGC TCT TGC TGG G (188 bp); for Ang-1 forward 5'-ACC CGA GCC TAT TCA CAG TA, reverse 5'-CAT TCA GTT TTC CAT GGT TT (272 bp); for HTNV S segment forward 5'-GGC CAG ACA GCA GAT TGG, reverse 5'-AGC TCA GGA TCC ATG TCA TC (878 bp), and for Tie-2 forward 5'-CAA ATT CAG TCC AGG ATG C, reverse 5'-AAT GTC ACC CAG GTC TTC (178 bp).

**Western blot analysis.** The infected or non-infected cells were harvested in lysis buffer and the protein content was quantified by Bradford assay (Bio-Rad). Western blotting was performed as described previously (Kim *et al.*, 2007). The primary antibodies were Ang-1, Tie-2 (Santa Cruz Biotechnology), and mouse anti-tubulin (Sigma-Aldrich). Anti-rabbit IgG-horseradish peroxidase (HRP) (Zymed) and anti-mouse IgG-HRP (Santa Cruz Biotechnology) were used as the secondary antibodies.

**Vascular permeability.** The permeability assay consisted of measuring the passage of HRP (Sigma-Aldrich) through confluent HUVEC monolayers grown on Transwell™ polycarbonate cell culture inserts (3 µm pore size and 12-well cluster plate; Costar) as described previously (Rabiet *et al.*, 1996). The permeability assay was performed on the day 2 p.i. The HUVECs used as the positive control were exposed to 1 U/ml of thrombin (Sigma-Aldrich) for 2 hrs and then were incubated with or without 250 ng/ml of Ang-1 (R&D Systems) for 2 hrs. The Transwell™ insert was transferred to a new plate containing serum-free medium. HRP (500 ng/ml) was added to the upper insert compartment and after 1 hr incubation at 37°C, the medium in the lower compartment was collected. HRP enzymatic activity was evaluated according to the manufacturer's instruction and the A<sub>470</sub> values were taken. The data were expressed in ng/ml of HRP that crossed the Transwell™ membrane.

**Plasma samples** of 17 HFRS patients, 3 non-HFRS patients (who exhibited symptoms similar to that of HFRS, but were serologically negative), and 5 healthy individuals were obtained from Soonchunhyang University Cheonan Hospital and Yeungnam University Hospital.

**Ang-1 ELISA.** Plasma Ang-1 levels were quantified by the Quantikine™ ELISA kit (R&D Systems). Three paired plasma samples obtained from acute and convalescent phase HFRS patients were also compared. ELISA was performed according to the manufacturer's protocol and the values A<sub>450</sub> were taken.

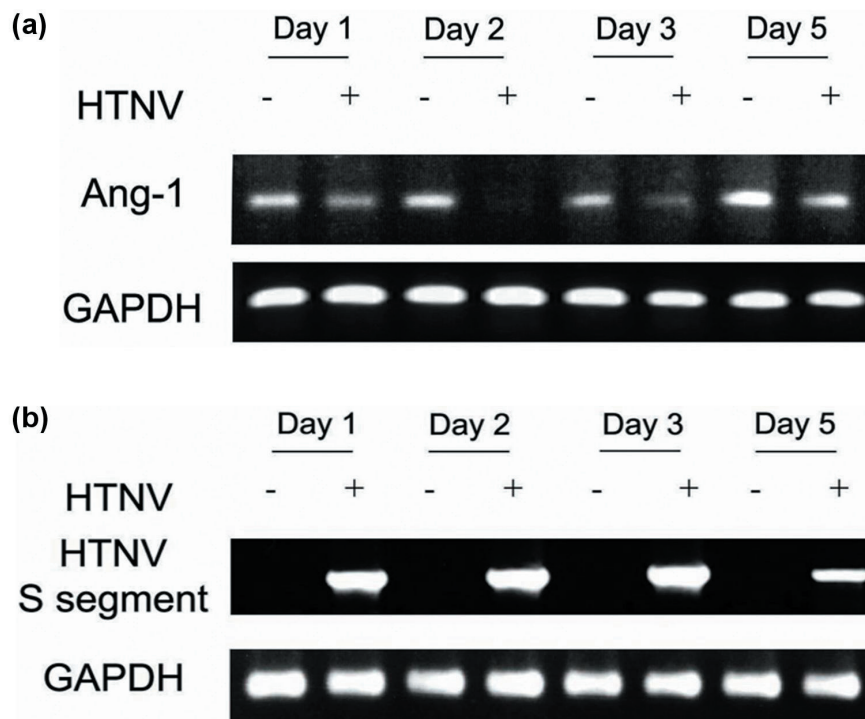
## Results and Discussion

### *HTNV suppresses Ang-1 expression*

One of the characteristics of HFRS caused by HTNV is an increased vascular permeability. Therefore, we examined the effects of HTNV on Ang-1 levels in HUVECs. To determine the influence of HTNV on Ang-1 transcription, RT-PCR was performed in the non-infected and HTNV-infected HUVECs. The Ang-1 mRNA levels were lower in HTNV-infected HUVECs than in control HUVECs from day 1 to day 5 p.i. The Ang-1 mRNA levels were markedly lower on day 2 and 3 p.i. (Fig. 1a). HTNV replication was confirmed by RT-PCR based on the detection of 897 bp S segment of HTNV RNA in HTNV-infected HUVECs. HTNV replication was very high at day 1–3 p.i. (Fig. 1b). Altogether, these findings suggested that Ang-1 mRNA levels were suppressed by the HTNV replication.

The indirect IFA was performed to investigate the localization and pattern of Ang-1 expression in HUVECs. The infected and non-infected HUVECs were immunostained

with antibodies against Ang-1 (red fluorescence) and HTNV (green fluorescence), and then counterstained with Hoechst 33258 (blue fluorescence) to identify nuclei. Control HUVECs exhibited very strong Ang-1 signals throughout the cytoplasm (Fig. 2a, upper panel). However, Ang-1 was weakly stained in HTNV-infected HUVECs, especially at day 2 and 3 p.i., what corresponded with the high rate of viral replication (Fig. 2a, lower panel). HTNV appeared as discrete pinpoint granules distributed in the perinuclear region of the cytoplasm, but it was absent from control HUVECs (Fig. 2a). Interestingly, the cells that did not express HTNV among the HTNV-infected HUVECs exhibited low Ang-1 signals (Fig. 2a, arrow heads) suggesting that the regulation of Ang-1 by HTNV may be both direct and indirect. Ang-1 protein levels were also evaluated by Western blotting. Two bands at approximately 55 K and 70 K were detected (Fig. 2b), and the 55 K band represented non-glycosylated Ang-1 and the 70 K band represented glycosylated Ang-1 (Hwang *et al.*, 2007; Davis *et al.*, 1996). The 55 K band was rarely detected at 1 day p.i. in both control and HTNV-infected HUVECs. The intensity of both bands was lower in HTNV-infected



**Fig. 1**

Ang-1 mRNA levels in HTNV-infected HUVECs

(a) Ang-1 mRNA. (b) HTNV S segment mRNA.

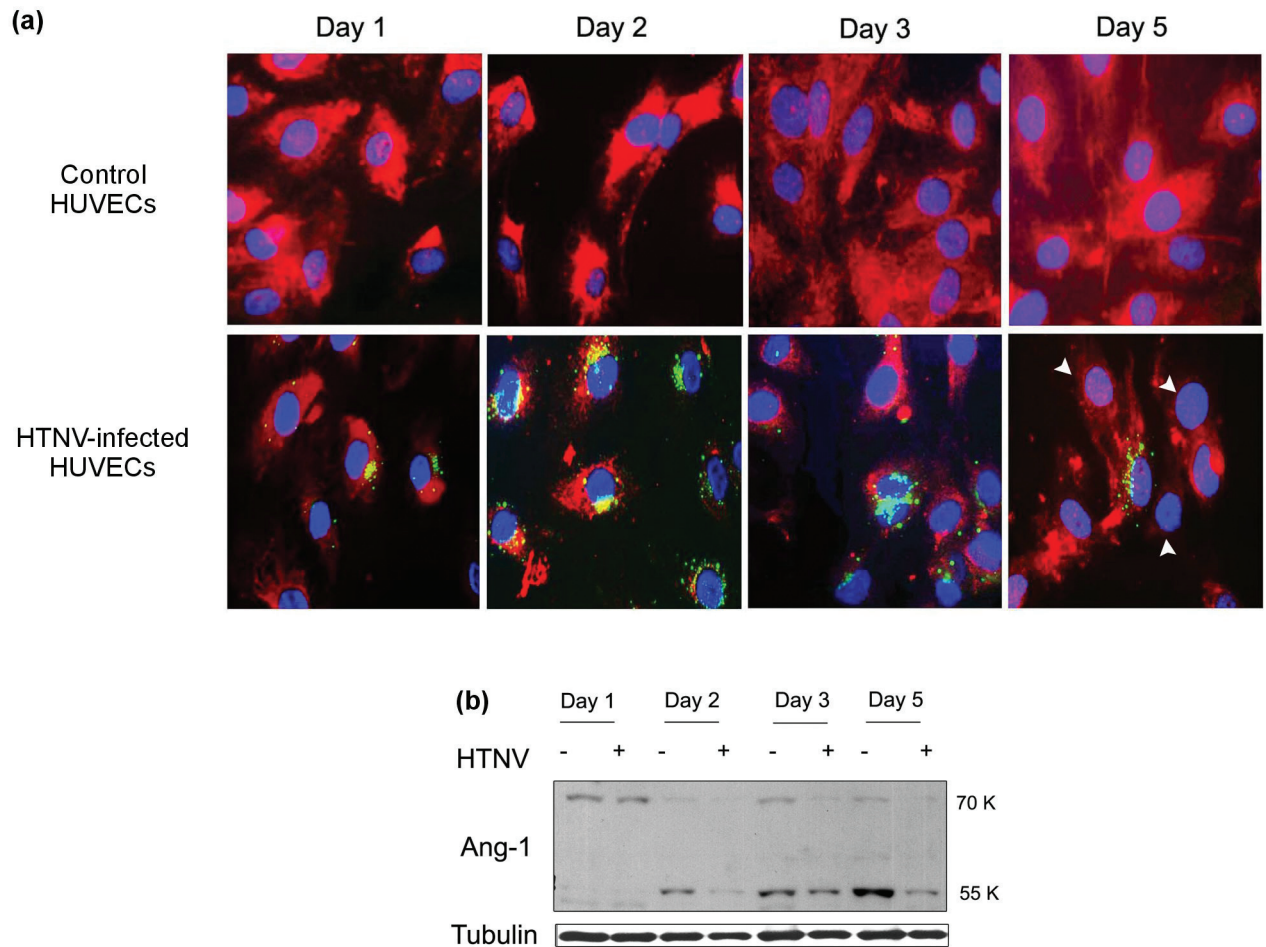


Fig. 2

**Ang-1 protein levels in HTNV-infected HUVECs**

(a) IFA: Ang-1 (red), HTNV (green), cell nuclei (blue). (b) Western blot analysis.

HUVECs than in the controls between days 2–5 p.i. (Fig. 2b). The data based on these experiments suggested that HTNV infection could reduce Ang-1 expression at both mRNA and protein levels.

*HTNV does not affect Tie-2 mRNA and protein levels*

As Tie-2 is a receptor for Ang-1, we investigated whether the mRNA and protein levels of Tie-2 were altered in HTNV-infected HUVECs. Tie-2 mRNA levels did not change after HTNV infection of HUVECs between days 1–5 p.i. (Fig. 3a). Tie-2 protein levels were also not significantly different between the infected and non-infected HUVECs during that time period (Fig. 3b).

*Ang-1 restores vascular permeability in HTNV-infected HUVECs*

To investigate the role of Ang-1 in the HTNV-infected HUVECs, a permeability assay was performed on the day 2 p.i. Thrombin was used as the positive control for this assay because it has been previously shown to increase endothelial permeability (Rabiet *et al.*, 1996). Treatment with thrombin induced a 1.8-fold increase in the permeability of HUVECs (Fig. 4). Addition of Ang-1 to thrombin-treated endothelial cells restored the normal levels of the endothelial permeability (Fig. 4). Endothelial permeability was increased 2-fold in HTNV-infected HUVECs relative to the control HUVECs. However, treatment of HTNV-infected HUVECs with

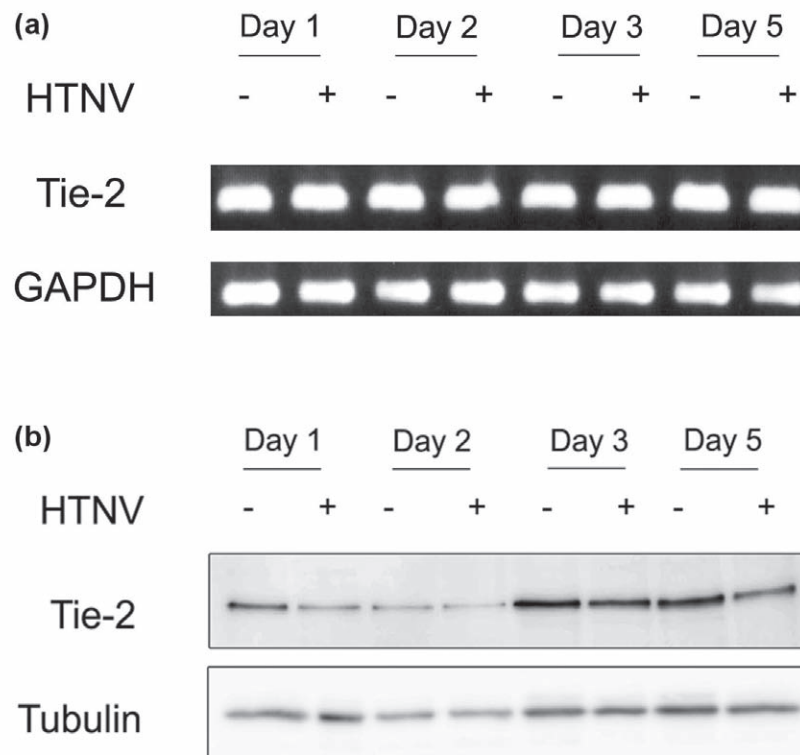


Fig. 3

**Tie-2 mRNA and protein levels in HTNV-infected HUVECs**

(a) RT-PCR. (b) Western blot analysis.

250 ng/ml Ang-1 decreased this permeability by 1.3-fold (Fig. 4). Our observations indicated that HTNV could decrease the Ang-1 expression, thus increasing the permeability, while intact Tie-2 was able to restore vascular permeability by the treatment with Ang-1 in HTNV-infected endothelial cells.

*Plasma Ang-1 levels are lower in HFRS patients compared with healthy individuals*

To determine the levels of Ang-1 in HFRS patients, we used an ELISA assay. We examined plasma Ang-1 levels from 17 HFRS patients, 3 non-HFRS acute febrile patients, and 5 healthy individuals. The mean plasma Ang-1 levels were 1,271 (range 19.5–3,169), 3,492 (range 2,407–5,307), and 3,226 pg/ml (range 1,974.5–5,307) in HFRS patients, non-HFRS patients, and healthy individuals, respectively. Therefore, the mean plasma Ang-1 levels of HFRS patients were lower than those of the other two groups (Fig. 5a). In addition, we also compared the Ang-1 levels of 3 paired plasma samples collected in acute and convalescent phase of HFRS

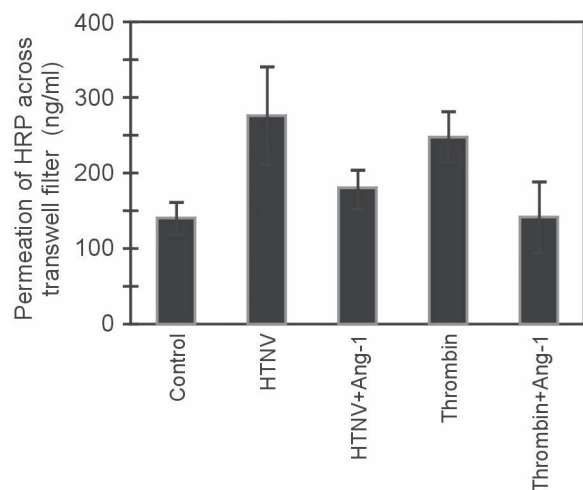


Fig. 4

**Permeability of HTNV-infected HUVECs**Mean  $\pm$  SD values from triplicate experiments.

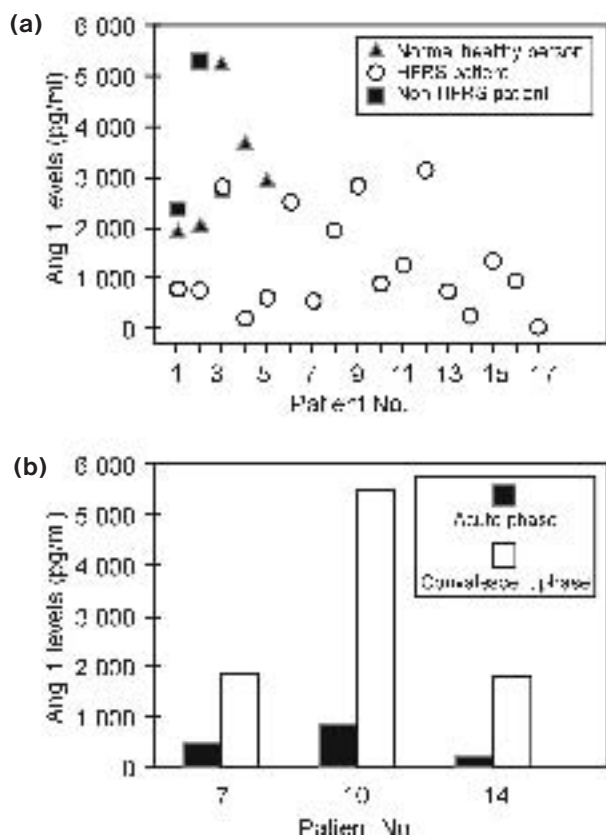


Fig. 5

**Plasma Ang-1 levels in HFRS patients, non-HFRS patients and healthy individuals assayed by ELISA**

(a) Ang-1 levels in individuals. (b) Ang-1 levels in paired plasma samples.

patients. The Ang-1 levels were 3.5–7.7-fold lower in the acute phase of the illness than in the convalescent phase (Fig. 5b). These observations are consistent with the drop of plasma Ang-1 levels upon HTNV infection in HFRS patients.

In summary, HTNV infection reduced the transcription and translation of Ang-1. In addition, the increased endothelial permeability by HTNV infection could be reduced by treatment with Ang-1. The plasma Ang-1 levels in acute phase of HFRS patients were markedly lower than those of healthy individuals or convalescent phase of HFRS patients. Therefore, a reduced expression of Ang-1 caused by HTNV infection could contribute to the endothelial permeability in HFRS patients and might be one of the factors responsible for the pathogenesis of HFRS.

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