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Hantaan Virus Reduces the von Willebrand Factor in Human Umbilical Vein Endothelial Cells

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To understand the molecular mechanism of hemorrhagic tendency represented in hemorrhagic fever with renal syndrome (HFRS), the effect of Hantaan virus (HTNV) on the von Willebrand factor (vWF) was observed in human umbilical vein endothelial cells (HuVECs). An immunofluorescence assay (IFA) showed a significant reduction of the vWF in the cytoplasm of HTNV-infected HuVECs. The amount of vWF protein in HTNV-infected HuVECs was reduced to 86, 49, 67, and 42% of those in control HuVECs at 1st, 3rd, 5th, and 7th days of post infection (d.p.i.), respectively. However, there were no significant differences in the vWF mRNA expression in both groups at all time courses by reverse transcriptase polymerase chain reaction (RT-PCR). The amounts of secreted vWF in the culture supernatants of the HTNV-infected HuVECs were 79, 87, 83, and 82% of those in control HuVECs at 1st, 3rd, 5th, and 7th d.p.i., respectively. These results indicated that the reduction of vWF by HTNV was regulated at post-transcriptional level and this might delay the coagulation process on the site of HTNV infection, thus leading to hemorrhage in HFRS.

Key Words: Hantaan virus, Endothelial cell, von Willebrand factor

INTRODUCTION

Hantaviruses cause two human disease, hemorrhagic fever with renal syndrome (HFRS), and hantavirus pulmonary syndrome (HPS), each able to manifest with varying degrees of severity (8,11,12,13). The etiologic agents responsible for HFRS include the Hantaan, Puumala, Dobrava, and Seoul viruses, and the etiological agents responsible for HPS include the Sin Nombre virus, the Andes virus, and the New York virus. However, the species of hantavirus are still growing. The major clinical findings associated with hantavirus diseases include vascular hemorrhage, kidney dysfunction, and shock (in HFRS) and acute respiratory distress accompanied by interstitial pneumonitis and pulmonary edema (in HPS). Although the major target organs of the two syndromes are different, common features of hantavirus diseases are acute thrombocytopenia and changes of permeability in the microvascular beds. The existence of these common features indicates that the vascular endothelium constitutes a prime target for viral infection (25). However, there were no evidences to suggest that the hantaviruses inflict direct damage to endothelial cells, either *in vivo* or *in vitro*, and the pathophysiological mechanisms of HFRS and HPS have not fully described yet.

Endothelial cells harbor several types of prothrombotic and antithrombotic molecules, including von Willebrand Factor (vWF), platelet activating factor (PAF), P-selectin, tissue factor (TF), plasminogen activator inhibitor-1 (PAI-1), nitric oxide (NO), prostacyclin (PGI2), adenosine, tissue-

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type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA), etc. (1). The balance between prothrombotic and antithrombotic molecules is crucial for the maintenance of homeostasis within the blood vessels.

The von Willebrand Factor (vWF) is a large multimeric protein, which is synthesized by megakaryocytes and endothelial cells (22,23). Although platelets also harbor vWF, the majority of vWF originates from the endothelial cells. The endothelial cells synthesize two forms of vWF: the vWF dimers, which are secreted into the plasma and the subendothelial matrix, and the granular vWF multimers, which are stored in the Weibel-Palade bodies (WPBs) for rapid mobilization in response to activating molecules, including thrombin (7). In case of vascular injury, vWF acts as a molecular bridge between platelets and the exposed subendothelium, leading to the formation of platelet thrombi and the subsequent arrest of bleeding (16,21). Another well-known aspect of vWF function is to carry and stabilize coagulation factor VIII, an anti-hemophilic factor, in plasma. Any quantitative or qualitative defect of vWF leads to von Willebrand disease (VWD), a genetic bleeding disorder. And also the thrombotic thrombocytopenic purpura (TTP) has implicated abnormal plasma von Willebrand factor (vWF)-cleaving metalloprotease activity (18). vWF expression is necessary not only for the targeting P-selectin and IL-8 to the WPBs but also for the adhesion of leukocyte through integrins (3,7,9,17). Therefore, vWF plays a prominent role in primary haemostasis and may modulate inflammatory process through its ability to target inflammatory mediator to the regulated secretion pathway of the endothelium.

In this study, we have observed the time-related effect of HTNV on vWF in human umbilical veins endothelial cells, in order to elucidate the mechanisms underlying the characteristic hemorrhage observed in HFRS.

MATERIALS AND METHODS

1. Cells and virus

The human umbilical vein endothelial cells (HuVECs) used in this study were purchased from Modern Tissue Technologies (MTT, Seoul, Korea), and were allowed to grow in EGM-2 (Cambrex, Walkersville, MD, USA) supplemented with 10% FBS, hFGT, R^3-igf-1, ascorbic acid, VEGF, hEGF, heparin, GA-1000, and hydrocortisone. Vero-E6 cells (ATCC CRL-1586) were grown in Dulbecco's Modified Eagles Medium (DMEM) with 10% FBS and antibiotics (Gibco BRL, Gaithersburg, MD, USA). These cells were incubated in a humidified atmosphere containing 5% CO_2 at 37℃. The Hantaan virus ROK 84-105 strain, which had been kindly provided by Dr. Ho Wang Lee, was propagated in Vero E6 cells for the preparation of a stock virus. HuVECs were grown for two days in 100 mm culture dishes, and then infected with HTNV at a multiplicity of infection (M.O.I) of 1.

2. Immunofluorescence staining

HuVECs were harvested and incubated overnight in 14-well slides and fixed for 10 min in cold acetone and dried in the air. Cells were blocked with 5% normal donkey serum for 30 min and then incubated for another 60 min at 37℃ in the presence of primary antibodies: serum taken from an HFRS patient and rabbit anti-von Willebrand factor antibody (Dako A/S, Glostrups, Denmark). After incubation, the cells were washed three times with PBS, and incubated for an additional 45 min in the presence of goat anti-human IgG-FITC and donkey anti-rabbit IgG-Cy3 (Jackson Laboratory, West Grove, PA, USA). Some slides were counterstained with Hoechst 33258 for nucleus and were observed under a fluorescence microscope (Zeiss, Oberkochen, Germany).

3. Western blot

For immunoblot tests, confluent monolayers of HuVECs were lysed with lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 5 mM DTT, 0.2 mM NaOrthovanadate, 100 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml leupeptin). The proteins were then separated on SDS-polyacrylamide gel, and then transferred to protran nitrocellulose transfer membranes (Schleicher & Schuell Bioscience, Dassel, Germany). These membranes were incubated with anti-vWF antibody (Dako A/S) and anti-tubulin antibody (Sigma, Saint Louis, MO, USA). The membranes

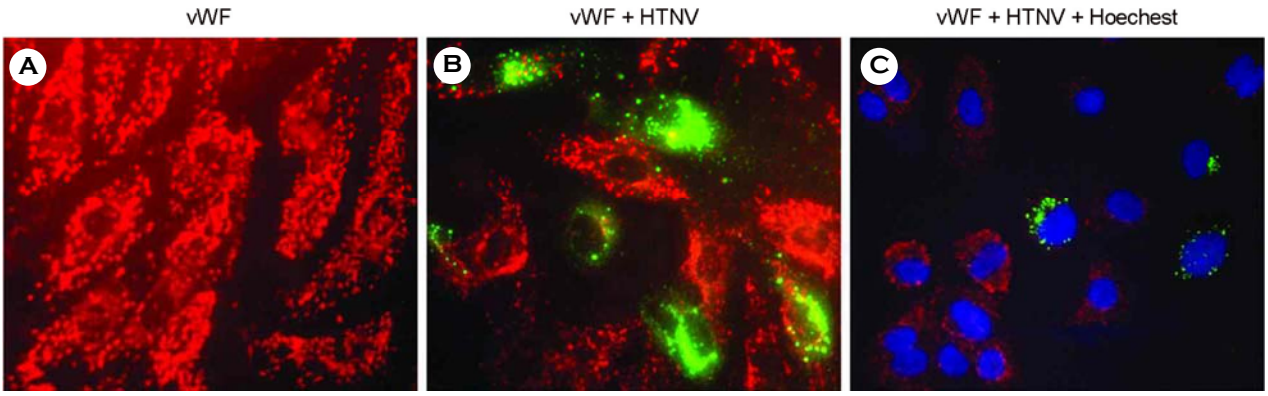

Figure 1. Expression of von Willebrand factor (vWF) in HuVECs. The vWF is shown in red, HTNV in green, and the cell nuclei in blue. The vWF and HTNV were double immunostained with rabbit polyclonal vWF antibody and HFRS patient serum as primary antibodies, followed by incubation with donkey anti-rabbit IgG coupled to Cy3, as well as goat anti-human IgG coupled to FITC, both of which were used as secondary antibodies in the control HuVEC **(A)** and HTNV-infected HuVEC **(B, C)**. Nuclei were counterstained with Hoechest 33258 **(C)**.

were incubated with goat-anti-rabbit Ig-HRP (Zymed, San Francisco, CA, USA) and developed with SuperSignal West Pico chemiluminescence agents (Pierce Biotechnology, Rockford, IL, USA), then exposed to X-ray film.

4. RT-PCR

Total RNA was extracted using RNA Bee (Tel-Test, Friendswood, TX, USA). Total RNA (1 μg) was reverse-transcribed in a 20 μl volume containing 15 units of AMV reverse transcriptase (Promega, Madison, WI, USA), 10 mM dNTP mix, and 1 μg/ml of oligo $(dT)_{15}$ primer. Each of the reverse transcription reaction was conducted for 15 min at 42℃, after which the reactions were discontinued via 5 min of denaturation at 95℃. The cDNA was subsequently amplified via PCR in a total volume of 50 μl, containing 5 μl of RT reaction buffer which consisted of 25 mM $MgCl_2$, 2.5 mM mixed dNTP, 25 pmole of each primer, and 2.5 units of DNA polymerase (BioTools, Madrid, Spain) in RNase-free distilled water. The sequences of primers used in these PCR experiments were as follows. For GAPDH, ACCCACTCCACCTTTS (sense) and CTCTTGTGCTC-TTGCTGGG (antisense), and the size of the product was 188 bp. For vWF, TGGTGAGGTGATTGTTCTGG (sense) and GATCCTAGTGGGGAATCCGG (antisense), the size of the product was 308 bp. For HTNV, AACCAGACAGC-AGATTGG (sense) and AGCTCAGGATCCATGTCATC (antisense), yielding an 878 bp product. After amplification, aliquots of the PCR reactions were separated on 1.5% agarose gel containing ethidium bromide.

5. Enzyme-linked immunosorbent assay (ELISA) for vWF

The amounts of secreted vWF in the supernatants of cultured HuVECs were determined with an ELISA assay as described by the manufacturer (Gradipore, Frenchs Forest, Australia). The culture media from the individual dishes were harvested and centrifuged for 15 minutes at 13,000 × g, and the supernatants were frozen at -80℃ until used. The optical densities were determined at 450 nm and converted to unit by following the instruction of manufacturer.

RESULTS

1. Immunofluorescence pattern of vWF in HTNV-infected HuVECs

The vWF and HTNV in the HuVECs were visualized via double immunostaining at 7th day post infection (d.p.i.). Most control HuVECs strongly expressed vWF in their cytoplasm (Fig. 1A). However, HTNV-infected HuVECs showed mush less vWF than in surrounding HTNV-uninfected cells (Fig. 1B). The HuVECs, which was counterstained with Hoechest 33258 after double immunostaining, showed obvious differences between the HTNV-infected and non-infected with regard to vWF expression (Fig. 1C).

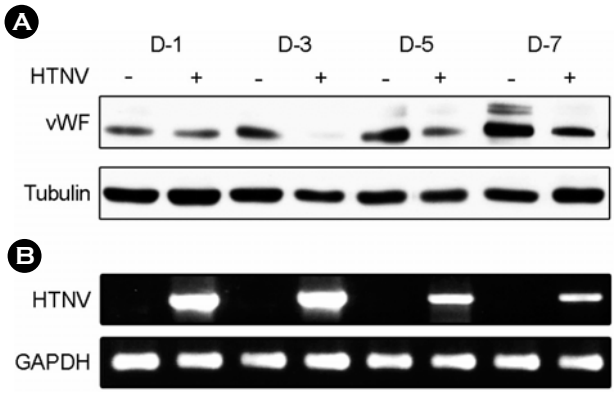

Figure 2. HTNV reduces vWF protein levels in the HuVECs. The control and HTNV-infected HuVECs were harvested at the indicated times (1, 3, 5, and 7 d.p.i.). **(A)** The cells were lysed in Laemmli's sample buffer, and the proteins were separated by SDS-PAGE and detected via immunoblotting using anti-vWF and anti-tubulin antibodies. **(B)** Total RNA was extracted from both the control and HTNV-infected HuVECs, and the levels of HTNV mRNA expression were assessed via RT-PCR.

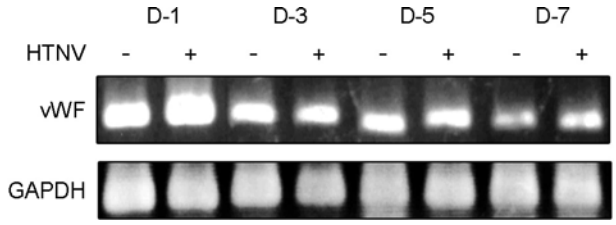

Figure 3. The expression of vWF mRNA was not altered by HTNV infection in HuVECs. HuVECs were either infected with HTNV or not infected, and the cells were harvested at the indicated times (1, 3, 5, and 7 d.p.i.). Total RNA was extracted from both the control and HTNV-infected HuVECs, and the levels of mRNA expression were assessed via RT-PCR.

2. HTNV reduced the vWF in HuVECs

The amounts of vWF protein in the HuVECs were quantified using Western blot analysis. The amounts of vWF increased gradually over time, but the amount of vWF in HTNV-infected HuVECs was reduced to 86, 49, 67, and 42% of those in control HuVECs at 1st, 3rd, 5th, and 7th d.p.i., respectively (Fig. 2A). Three different bands of vWF were prominent in control HuVECs at 7th d.p.i., but not seen in 1st and 3rd d.p.i. in both groups (Fig. 2A). To compare the viral expression with vWF protein expression, S segment of HTNV genome was detected with RT-PCR and HTNV RNA was highly expressed from 1st to 3rd d.p.i. and then decreased (Fig. 2B). The expression of vWF protein in HTNV-infected HuVECs showed inverse relation with HTNV RNA expression (Fig. 2A, 2B).

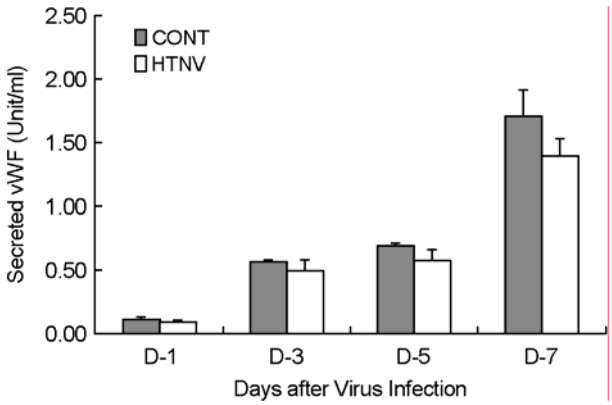

Figure 4. HTNV reduced the levels of vWF secretion. The culture supernatants were harvested at the indicated times (1, 3, 5, and 7 d.p.i.) from both the control and HTNV-infected HuVECs, and the amounts of vWF were quantified by ELISA. Each value represents mean ± SD of three independent experiments.

3. vWF mRNA expressions were not significantly different

In order to determine whether the reduction of vWF protein was regulated at the transcriptional level or not, we observed the levels of vWF mRNA in both control and HTNV-infected HuVECs by RT-PCR. The difference of the vWF mRNA expression between control and HTNV-infected HuVECs was not significant at each time points (Fig. 3).

4. Secreted vWF levels were reduced in HTNV-infected HuVECs

The culture supernatants of the control and HTNV-infected HuVECs were collected from 1st to 7th d.p.i., in order to measure the amount of secreted vWF at each time points. The quantities of secreted vWF were 0.09, 0.55, 0.68, 1.70 unit/ml from control supernatants and 0.07, 0.48, 0.56, and 1.39 unit/ml from HTNV supernatant at 1st, 3rd, 5th, and 7th d.p.i., respectively. The amounts of vWF were very low at 1st d.p.i. and gradually increased over time in both control and HTNV supernatants (Fig. 4). The reduction rate of secreted vWF by HTNV was 79%, 87%, 83%, and 81% at 1st, 3rd, 5th, and 7th d.p.i., respectively (Fig. 4).

DISCUSSION

The primary hematological characteristics of HFRS include hemorrhage, thrombocytopenia, and disseminated intravascular coagulation (DIC) (4,6,15). According to the clinical and laboratory findings, hemorrhage appears between 2 to 5 days after the emergence of fever. Also, the platelet counts and coagulation factors, including factor II, V, VIII, IX, and X decreased in the early stages of disease (14,20). However, there were no evidences of the direct endothelial cell damage inflicted by Hantaviruses, both *in vivo* and *in vitro*. Recently, several studies have used endothelial cells to characterize the pathogenesis of HFRS and HPS, and it is known that HTNV can provoke changes in a variety of molecules, including interferon-related molecules, chemokines, and cell surface receptors, etc (2,5,10,19,20,24).

One of the most significant findings in this study was that Hantaan virus inhibits the accumulation of vWF in endothelial cells (Fig. 1, Fig. 2A). Especially, vWF was greatly reduced at 3rd d.p.i. when the viral RNA expression reached the peak and the expression of vWF showed inverse relation with HTNV RNA expression (Fig. 2A, 2B). To find out whether the change of vWF was regulated at the transcriptional level or not, we performed RT-PCR and there were no significant differences between control and HTNV-infected HuVECs and this result was consistent with the results of a previous study (20) (Fig. 3). In addition, the ELISA results revealed that the secretion of vWF gradually increased over time in both group but the amount of secreted vWF was approximately 20% lower in HTNV-infected HuVECs than in control HuVECs (Fig. 4). Those results represented that HTNV had inhibited the accumulation of vWF inside the endothelial cells more than the secretion of vWF to the outside of the cells.

The molecular weight of mature vWF monomer is 220 kDa. However, others forms of vWF (pre-pro vWF, pro-vWF, dimmer, oligomer, multimer) also exist because the synthesis of vWF includes several processing, such as cleavage of signal peptide upon translocation into the endoplasmic reticulum (ER), N-linked glycosylation and dimerization in ER, sulfation, O-glycosylation and high-mannose oligosaccharide processing in Golgi apparatus. The molecular weight of stored vWF in WPBs is 220 kDa and the secreted vWF consists with 220 kDa and 275~320 kDa forms. Therefore, upper two thin bands detected in Western blot as the secreted forms of vWF (Fig. 2A) were not seen in 1st and 3rd d.p.i. and the amount of secreted vWF was also extremely low at 1st d.p.i. in both groups (Fig. 2A, Fig. 4). The secretion of vWF increased from 3rd to 7th d.p.i. in both groups (Fig. 4), but the upper bands were still weak until 5th d.p.i. in both groups (Fig. 2A). From those results, we expected that the secretion of vWF was prior to the accumulation in HuVECs. Hantaan virus is an enveloped virus with two glycoproteins (G1 and G2). In the HTNV replication processes, the synthesis of viral glycoproteins takes place in ER and Golgi. Therefore, we could speculate that the synthesis of vWF might be inhibited by competition with HTNV in ER and Golgi and it might overcome when the HTNV replication had decreased at late time.

Since vWF is one of very important factor in primary haemostasis together with platelet, the lack of vWF in the HTNV-infected endothelial cells might involve in the mechanisms underlying the hemorrhagic tendencies associated with HFRS.

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