大韓바이러스學會誌：第28卷 第4號 1998
J. Korean. Soc. Virology, Vol. 28, No. 4, 1998

Effects of Hantaan Virus and IFN-γ on Induction of Surface ICAM-1 in Primary Cultured Human Nasal Epithelial Cells and Human Lung Fibroblasts

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=Abstract=

The primary culture of human nasal epithelial cells was performed using the inferior nasal turbinate tissues, and infected with Hantaan virus to examine the hypothesis of airborne transmission of Hantaan virus in humans. The primary culture cells were identified as epithelial cells by morphologic and immunologic analyses. The viral antigens were detected in the primary human nasal epithelial cells infected with Hantaan virus by immunofluorescence staining.

The ICAM-1 induction by Hantaan virus or IFN-γ was examined in the primary human nasal epithelial cells and human lung fibroblasts (WI-38). Hantaan virus induced the surface ICAM-1 in WI-38 cells in a time-dependent manner, and IFN-γ induced the surface ICAM-1 in a dose-dependent manner in HNEC and WI-38 cells.

These results revealed that the human nasal epithelial cells are susceptible to Hantaan viral infection supporting the hypothesis of airborne transmission of Hantaan virus in humans. The human lung fibroblasts also might have an important role in the pathogenesis of Hantaan virus through the induction of ICAM-1.

Key Words: Hantaan virus, ICAM-1, Human nasal epithelial cell, Human lung fibroblast

INTRODUCTION

Hantaan virus is an etiological virus of hemorrhagic fever with renal syndrome [11] and a prototype virus of Hantavirus genus in Bunyaviridae [26, 27]. Not only the pathophysiological mechanism in the human body but also the biochemical changes in the cells by Hantaan virus are not well understood because there are no proper animal models to study the pathogenesis of Hantaan virus. Even though rodents are the main host of the Hantaan virus, they do not develope the same disease which occurs in humans. Recently, there are many attempts to reveal the host-viral interaction using a cell culture system. Hantaan virus can infect several kinds of cell lines such as lung carcinoma cell [4], kidney epithelial cell [16], endothelial cell [20], T cell [8], fibroblast [18, 19], hepatocarcinoma cell [9], etc. The infection route of Hantaan virus is believed to be through aerosol transmission in rodents [17], but there is only the epidemiologic evidence in humans [30]. There are no reports concerning the human nasal epithelial cells which are encountered first when the Hantaan virus invades the human body through aerosol

접수 : 1998년 9월 22일

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transmission. Therefore, we prepared human nasal epithelial cells using a primary culture and tried to infect the cells with Hantaan virus.

Intercellular adhesion molecule-1 (ICAM-1) is a cell surface glycoprotein expressed on many cell lineages and it belongs to the immunoglobulin superfamily [3, 23]. It has important roles in the process of inflammation. It functions in intercellular adhesion reactions by binding to its ligand, LFA-1(CD11a/CD18), a member of the integrin family [15]. ICAM-1 is expressed in several kinds of cells such as endothelial cell, monocyte, keratinocyte, epithelial cell, B and T lymphocytes. ICAM-1 is highly inducible in some cell types by proinflammatory cytokines [5, 14, 21, 24, 29]. IFN-γ induces ICAM-1 on primary human tracheal epithelial cells [14] and on human umbilical vein endothelial cells [22]. Hantaan virus is known to induce ICAM-1 expression in human umbilical vein endothelial cells and may play a key role in adhesion and extravasation of inflammatory cells [1]. But there are no reports about the ICAM-1 expression in other cell types which can be infected with Hantaan virus. We examined the surface ICAM-1 expression by Hantaan virus or IFN-γ in primary cultured human nasal epithelial cells and human lung fibroblasts (WI-38).

MATERIALS AND METHODS

Virus, cells, and media

The Hantaan virus ROK-84-105 strain was propagated in VeroE6 cells (ATCC CRL-1586). After 7 days of viral infection, culture supernatants of VeroE6 cells were harvested and centrifuged at 1,730 *g* for 30 min. Aliquots of the centrifuged supernatants were stored at -70℃ until use. Mongolian gerbil fibroblast (MGF) established by primary culture from Mongolian gerbil embryo was provided by Mogam Biotechnology Research Institute. WI-38 cell line (ATCC CCL-75) was purchased from ATCC (American Type Culture Collection, Maryland, USA). Bovine pulmonary artery endothelial cell (CPAE: ATCC CCL-209) was kindly provided by Professor Kyu-Won Kim at Pusan National University. VeroE6, MGF and WI-38 cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100U/ml penicillin, 100 μg/ml streptomycin, and 0.25μg/ml Fungizone). CPAE was grown in DMEM supplemented with 20% fetal bovine serum and antibiotics. Human nasal epithelial cells (HNEC) were prepared from inferior nasal turbinate tissues using a primary culture method. HNEC was grown in Medium 154 (Cascade Biologics, Oregon, USA).

Primary culture of human nasal epithelial cell

HNEC were cultured from inferior nasal turbinate tissues which were obtained from partial turbinectomy of septal deviation patients. HNEC were isolated by method of monolayer culture of dissociated cells described by Look, Kenney and Devalia [14, 7, 2] with modifications. Briefly, immediately after surgery the tissues were placed in phosphate-buffered saline (PBS) with antibiotics (100U/ml penicillin, 100μg/ml streptomycin, and 0.25μg/ml Fungizone) and transported to the laboratory. The tissues were washed three times in PBS containing antibiotics. Excessive connective tissues were cut away and the remaining epithelia were cut into pieces approximately 5 x 5mm in size. The pieces were placed in the dispase solution (neutral protease: grade II, Boehringer Mannheim, Germany) for 90 min in 37℃, and then the epithelial cells were gently scraped away from the connective tissues with a scalpel blade and placed in a 50 ml centrifuge tube containing PBS. The cell suspension was passed through 200 mesh (Bellco, USA) and centrifuged at 300 *g* for 10 min and then the pellet was resuspended in Medium 154. The cells were cultured in 6 well tissue culture plates and incubated in 5% CO_2 at 34℃. The cells which did not attach to the plates were re-

moved and the remained cells were replenished with fresh medium 24 h later.

Confirmation of epithelial cell identity

The identity of primary HNEC was confirmed by light microscopy, electron microscopy, and immunofluorescence staining. For electron microscopy, cultured cells were fixed in 2.5% glutaraldehyde in 0.2M phosphate buffer, pH 7.3 for 5 min and washed with the same buffer. The cells were fixed in 1% osmium tetroxide for 30 min and dehydrated through graded ethanol series prior to being embedded. The cells were embedded in epoxy resin during 48 h at 60℃. Sections (50～70nm) were cut both horizontally and vertically followed by staining with uranyl acetate and lead citrate.

For immunofluorescence staining, cells were harvested and cultured overnight in 14 well slides. The slides were washed with PBS and fixed in cold acetone for 10 min and then incubated for 60 min at 37℃ in the presence of specific primary antibodies: anti-cytokeratin pan and anti-cytokeratin 8 (Boehringer Mannheim, Germany) directed against epithelial cells; anti-vimentin (Sigma, St. Louis, USA) directed against the cells originated from mesoderm such as fibroblasts; anti-von Willebrand factor (Boehringer Mannheim, Germany) directed against endothelial cells. Following incubation, the cells were washed three times with PBS and further incubated for 45 min in the presence of secondary antibodies conjugated with FITC. After incubation, the slides were washed twice with PBS and once with distilled water, then dried in the air. The slides were mounted with mounting medium and observed by fluorescence microscope (Zeiss, Axiophot, Germany). MGF and CPAE were used as positive control cells for vimentin and von-Willebrand factor, respectively.

Detection of Hantaan virus in human nasal epithelial cells

HNEC were infected with Hantaan virus at 0.2 M.O.I. and incubated for 5 days. The cells were harvested and cultured in 14 wells slides overnight for immunofluorescence staining as described above. Diluted hemorrhagic fever with renal syndrome patient serum was used for as a primary antibody. The presence of Hantaan viral antigens in HNEC was observed with fluorescence microscope.

Virus and Interferon-γ treatment

HNEC and WI-38 were grown to the monolayers in 6 well tissue culture plates and infected with Hantaan virus at 0.01 M.O.I or treated with 0.01, 0.1, 1ng/ml of recombinant human IFN-γ (Endogen, USA). The cells were harvested 24 h later and used for FACS analysis. In addition, the cells were harvested 24, 48, 72, 96 h after the Hantaan virus infection or treatment of 1 ng/ml of IFN-γ.

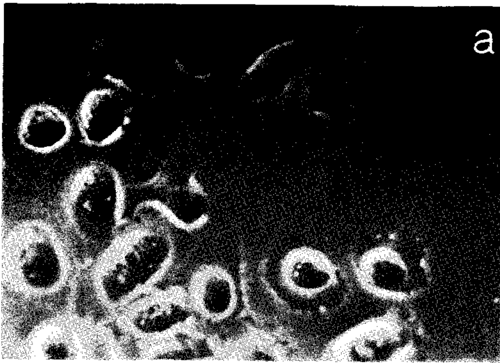
Fluorescence-activated cell sorting (FACS) analysis

After the treatment of virus or IFN-γ, the cells were harvested with trypsin-EDTA and washed with PBS. The cells were stained with ICAM-1-PE (Becton Dickinson, USA) for 40 min at 4℃. Monoclonal mouse IgG2a-PE was used for a negative control antibody. After washing with PBS, the mean intensity of fluorescence (MIF) of ICAM-1 on the cells was measured by ELITE (Coulter, USA) FACS analysis machine.

RESULTS

Primary culture and identification of HNEC

The isolated cells from inferior nasal turbinate tissues proliferated and formed several groups of small colonies at 2～3 days (Figure 1a) and formed monolayers at 5～7 days (Figure 1b). The primary culture cells were confirmed morphologically and immunologically as a human nasal epithelial cell. Inverted phase-contrast microscopy of the primary culture cells re-



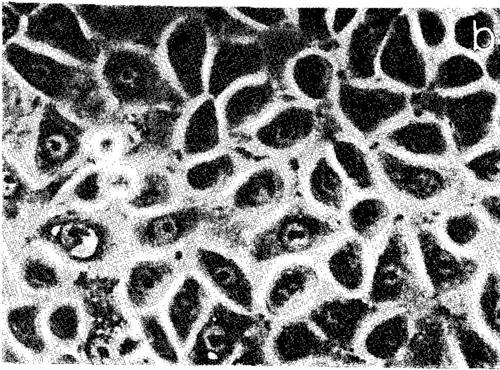


Figure 1. Photomicrograph of cultured human nasal epithelial cells (HNEC) 3 days (a) and 7 days (b) after primary culture from nasal turbinate tissue.

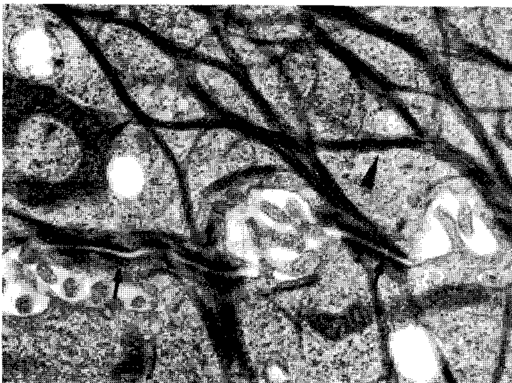
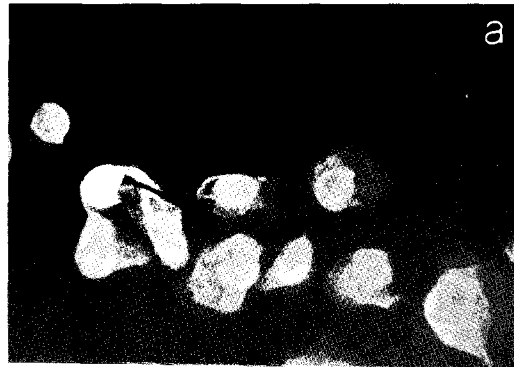


Figure 2. Transmission electron micrograph of horizontal section of cultured human nasal epithelial cells at confluence. Tonofilaments (arrowhead) and desmosomes (arrow), specific microstructures of epithelial cells, are observed.



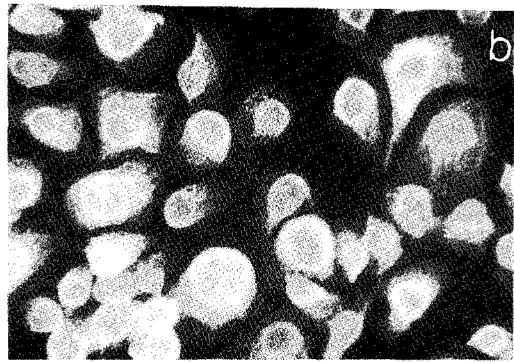


Figure 3. Immunofluorescence staining of human nasal epithelial cells for cytokeratin-pan (a) and cytokeratin No. 8 (b).

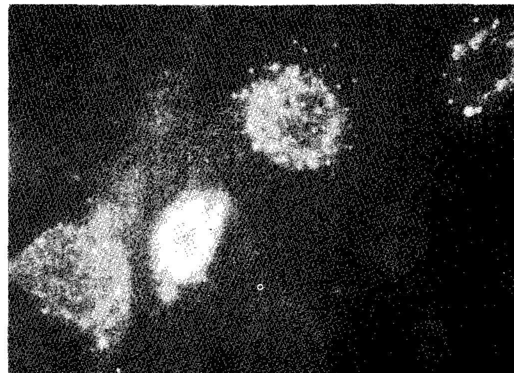


Figure 4. Immunofluorescence photomicrograph of HNEC infected with Hantaan virus. The discrete intracytoplasmic fine granular pattern of viral antigens were observed at 5 days postinfection.

vealed a uniform monolayer of polygonal cells that were homogeneous in shape and free of fibroblast contamination (Figure 1b). To investigate the nature of the cells, we performed transmission electron microscopy. Tonofilaments and desmosomes, the typical microstructures of epithelial cell, were detected in the cells (Figure

2). In immunofluoresecence staining, the primary cultured cells reacted with anti-cytokeratin pan (Figure 3a) and anti-cytokeratin 8 antibodies (Figure 3b) but did not react with antibodies for von-Willebrand factor and vimentin (data not shown). MGF and CPAE did not react with anti-cytokeratin pan and anti-cytokeratin 8 antibodies but reacted with antibodies for vimentin and von-Willebrand factor, respectively (data not shown). These results showed that the primary culture cells were epithelial cells and free from endothelial cell or fibroblast contamination.

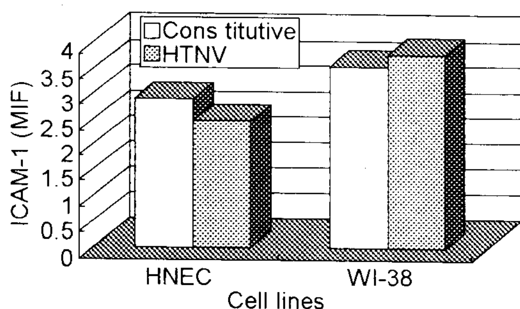


Figure 5. Expression of surface ICAM-1 after the infection of Hantaan virus in HNEC and WI-38 cells. 24 hours after infection, the cells were harvested and stained with anti-ICAM-1-PE antibody. Mean intensity of fluorescence (MIF) of ICAM-1 were measured by FACS.

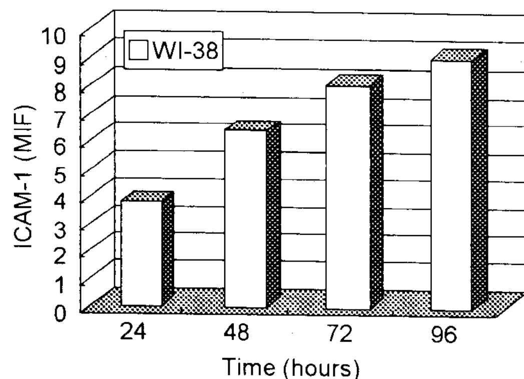


Figure 6. ICAM-1 expression in WI-38 cells according to the time course treatment of Hantaan virus. The cell were harvested at 24, 48, 72 and 96 hours postinfection of Hantaan virus.

Confirmation of Hantaan virus infection in HNEC

Infection of Hantaan virus in HNEC were confirmed by indirect immunofluoresecence staining with hemorrhagic fever with renal syndrome patient serum. Intracytoplasmic hantaan viral antigens were detected in HNEC at 5 days postinfection (Figure 4).

Surface ICAM-1 expression in the HNEC and WI-38 cells

The effects of Hantaan virus or IFN-γ on the expression of surface ICAM-1 were measured in HNEC and WI-38 cells using a FACS ma-

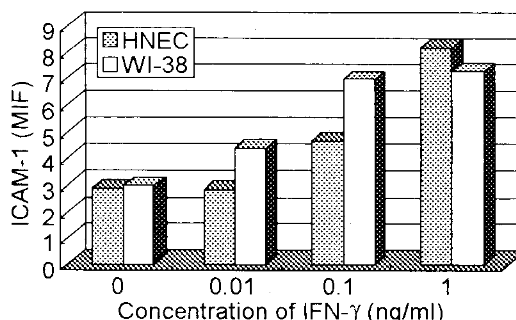


Figure 7. Mean intensity of fluorescence of ICAM-1 in HNEC and WI-38 cells after the treatment of interferon-γ. The cells were treated with 0, 0.01, 0.1, 1ng/ml of IFN-γ for 24 hours.

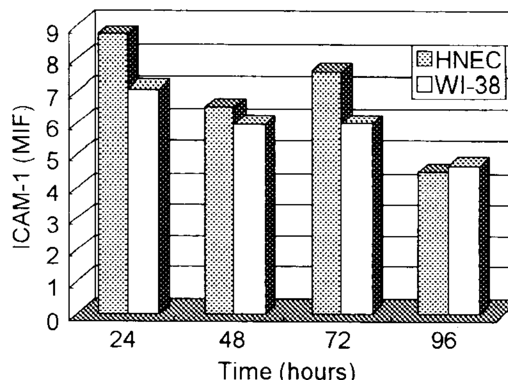


Figure 8. ICAM-1 expression in HNEC and WI-38 cells according to the time course treatment of interferon-γ. The cell were treated with 1ng/ml of IFN-γ for 24, 48, 72 and 96 hours.

chine. After infection or treatment of IFN-γ, the cells were harvested and stained with anti-ICAM-1-PE antibody and then analyzed with FACS. In HNEC and WI-38 cells, 24 h treatment of Hantaan virus showed little difference in induction of surface ICAM-1 compared with the constitutive expressions in each cell lines (Figure 5). The expression level of ICAM-1 increased according to the time course treatment of Hantaan virus in WI-38 cells (Figure 6). Twenty four hour treatment of IFN-γ showed the increase of ICAM-1 in a concentration-dependent manner in both cell lines (Figure 7). In the time course treatment of IFN-γ, the peak expression of ICAM-1 was at 24 h, and then decreased in both cell lines (Figure 8).

DISCUSSION

Previous studies suggest that aerosolized virus may be an important route of transmission for Hantaan virus infection [12, 13]. Epidemiological studies by Xu *et al.* [30] provided circumstantial evidence of airborne transmission in humans, and Nuzum E.O *et al.* [17] proved air transmission of Hantaan virus in Wistar rats using small-particle aerosols. To prove these hypothesis in human is impossible, it was attempted to infect Hantaan virus to the primary cultured human nasal epithelial cells.

Firstly, human nasal epithelial cells were prepared from inferior nasal turbinate tissues using a primary culture method called monolayer culture of dissociated cells. Usually this method has been used for studying nasal epithelial cell character or behavior because it is possible to obtain pure nasal epithelial cells without contamination of other cell types. Also a specific serum-free medium (Medium 154) was used to promote the growth of epithelial cells and suppress the growth of fibroblasts. After confirmation of the primary cultured cells as a pure human nasal epithelial cell, the cells were infected with Hantaan virus and then Hantaan virus antigens were detected by indirect immunofluorescence staining. A distinct small discrete granular pattern of Hantaan viral antigen was detected at 5 days postinfection in the cells (Figure 4). These results suggested that human nasal epithelial cells were susceptible to Hantaan viral infection and it showed the possibility of aerosol transmission through the human nose.

Pulmonary edema and congestion is one of the pathologic findings in hemorrhagic fever with renal syndrome [10]. And also the main pathologic findings in the hantavirus pulmonary syndrome caused by Sin Nombre virus, a member of hantavirus genus, are alveolar and interstitial pulmonary edema, pleural effusions, and lymphoid cell infiltration of pulmonary interstitium [6]. It means that there is an infection in pulmonary interstitium by Hantaan virus or Sin Nombre virus, which can attract lymphocytes. Pulmonary edema, either in interstitium or alveolar, may be caused by vasodilatation induced by chemical mediators from infiltrate lymphocytes. The main components of pulmonary interstitium are fibroblasts and capillaries. The human lung fibroblasts (WI-38) and human endothelial cells are known to be infected with Hantaan virus [1, 18, 20], so they might play an important role in the pathogenesis of hemorrhagic fever with renal syndrome. Chung *et al.* [1] already demonstrated that Hantaan virus induces ICAM-1 expression in the human umbilical vein endothelial cells, and also the mononuclear cells from human peripheral blood attach to the endothelial cells infected with Hantaan virus.

To investigate the expression of ICAM-1 by Hantaan virus or IFN-γ, two types of human cells were used, primary cultured human nasal epithelial cells (HNEC) and human diploid lung fibroblasts (WI-38) in this experiment. IFN-γ was used because it is produced by T lymphocytes or NK cells sensitized by viral antigens and induces ICAM-1 in some cell types [14, 25]. ICAM-1 increased in a time-dependent manner in WI-38 cells when treated with Han-

taan virus (Figure 6). It means that the expression of ICAM-1 is proportional to the production of viral progeny. Twenty four hour treatment of IFN-γ in HNEC and WI-38 had significant effects on the induction of ICAM-1 in a concentration-dependent manner (Figure 7). This means that if there are enough virus to sensitize T or NK cells, human nasal epithelial cells or human lung fibroblasts can induce ICAM-1 through IFN-γ in vivo. But the amount of hantaan viral antigen in HNEC was small compared with VeroE6 or WI-38 cells. We could detect a lot of hantaan viral antigens at 14 days postinfection in VeroE6 or WI-38 cells [18], however the viral antigens in HNEC disappeared spontaneously at 10 days postinfection. Therefore it is assumed that even though Hantaan virus could infect HNEC, and ICAM-1 could be induced by IFN-γ in HNEC, the nasal epithelial cells could not produce enough virus to evoke significant pathological changes in the nose. As it was found that Hantaan virus or IFN-γ can induce ICAM-1 in the human lung fibroblast, we hypothesize that not only the endothelial cells but also the lung fibroblasts infected with Hantaan virus might play an important role in lung edema and congestion through the attraction of lymphocytes via ICAM-1 expression.

SUMMARY

We have prepared human nasal epithelial cells (HNEC) from inferior nasal turbinate tissues using primary culture method called monolayer culture of dissociate cells. The primary cultured cells showed polygonal shape and typical microstructures of epithelial cell such as tonofilament and desmosome. The cells were also characterized as epithelial cells by immunological method using a panel of monoclonal antibody against epithelial cell. The HNEC were infected with Hantaan virus and the viral antigens were detected by immunofluorescence staining. That result supports the possibility of airborne transmission of Hantaan virus in human.

ICAM-1 induction by Hantaan virus or IFN-γ was examined in human nasal epithelial cells (HNEC) and human lung fibroblasts (WI-38). Hantaan virus induced surface ICAM-1 expression in WI-38 cells in a time-dependent manner, and IFN-γ induced surface ICAM-1 in HNEC and WI-38 cells in a dose-dependent manner. Therefore the infection of Hantaan virus in human lung fibroblasts may play an importnat role in the pathogenesis of hemorrhagic fever with renal syndrome via ICAM-1.

Acknowledgement

The authors would like to thank Professor Kim Yong-Dae for the supply of nasal turbinate tissues and Kim Ja-Eun for the assistance of this experiment. This research was supported by the Yeungnam University Research Grants in 1997.

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