HS N-sulfation and iduronic acids play an important role in the infection of Respiratory Syncytial Virus \textit{in vitro}

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\textbf{Abstract.} – BACKGROUND: As a member of Glycosaminoglycans (GAGs), heparan sulfate (HS) are sulfated to varying extents and used by a large number of viruses to initiate infection, including respiratory syncytial virus (RSV). Heparinases I, II, III can remove N-sulfation and iduronic acids units of HS, and low-molecular-weight heparin (LMWH) has a very similar structure to that of HS. AIM: The tropism of RSV for different cell lines and the efficiency of Heparinases and LMWH in inhibiting RSV infection were estimated in this study.

MATERIALS AND METHODS: Hela, Hep-2, HEK293 and Lo2 cell lines were pretreated with heparinases I, II, III and LMWH, and the cells were infected by RSV in vitro. RSV infectivity was determined by flow cytometry and western-blot.

RESULTS: All cells were susceptible to RSV except Lo2. Heparinases I, II, III and LMWH treatments reduced the susceptibility of Hep-2 cells to RSV infection. For HEK-293 heparinase II and III treatment could reduce RSV infection. All enzymes could not change the susceptibility of Hela cells to RSV infection.

CONCLUSIONS: These findings suggest that the heterogeneity of HS especially for rich N-sulfation and iduronic acids may play an important role in RSV infection in some mammalian cells.

Key Words: Respiratory syncytial virus, Heparan sulfate, Infec
tio, N-sulfation, Iduronic acids units.

\section*{Introduction}

Human respiratory syncytial virus (RSV) is an enveloped, negative-strand RNA virus in the Pneumovirinae subfamily of the Paramyxoviridae family. Human RSV causes severe lower respiratory tract infection, particularly in infants and older adults\cite{1,2}. RSV infection is mediated partly by an initial interaction between attachment protein (G) and a highly sulfated heparin-like glycosaminoglycans (GAGs) located on the cell surface\cite{3,4}. However, the effective agents involved in RSV infection are still poorly understood. As a member of GAGs, heparan sulfate (HS) are sulfated to varying extents and used by a large number of respiratory tract viruses to initiate infection of immortalized cell lines. HS chain is generally attached to core proteins, forming heparan sulfate proteoglycan (HSPG). Previous studies have suggested that the presence of cell surface GAGs containing iduronic acid, like heparan sulfate and chondroitin B, is required for efficient RSV infection in cell culture\cite{5}. In this study different tissue-derived cell lines were pretreated with heparinases and low-molecular-weight heparin (LMWH) to evaluate the role of HS N-sulfation and iduronic acids units in the RSV infection. Heparinases I, II, III can remove N-sulfation and iduronic acids units of HS, and LMWH is a negatively charged glycoprotein, which has a very similar structure to HS\cite{6}. Whether Heparinases and LMWH might inhibit positively charged respiratory syncytial virus (RSV) infection was investigated.

\section*{Materials and Methods}

\textbf{Cells and Virus}

The long strain of human RSV was purchased from the viral institute of Chinese Academy of Preventive Medical Science (Beijing, China). The cell lines including Hep-2 (human lung ep-
ithelial tumor cell line, CCL-23, ATCC), HEK293 (human embryonic kidney cell line, CRL-1573, ATCC), Lo2 (human liver cells, USA Sciencell) and Hela (human cervix carcinoma cell line, CCL-2, ATCC) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS).

RSV Infection
Infectivity of virus stocks was determined on Hela cell monolayers and calculated using the method of Reed and Muench, and titers were reported as 50% tissue culture infectious doses. For titration, 10-fold dilutions of the virus were made in RPMI-1640, 2% FBS. Twenty microliters of each dilution was added to cells in a 96-well plate. After incubation for 1 h with rocking, inocula were removed and replaced by RPMI-1640 containing 2% FBS. Cells were incubated for 24 h at 37°C before observation. Endpoint titers were determined by the method of Reed and Muench (7,8).

Heparinase and LMWH Treatment
Hep-2, Lo2 and HEK-293 monolayers were treated with Heparinase I, II, III and LMWH at 37°C for 1 h, then incubated with RSV (100TCID50) at 37°C for 1 h. At last, the supernatant was removed and replaced by RPMI-1640 containing 2% FBS.

Flow Cytometry
Cells were fixed and labeled by use of anti-RSVF primary antibody and fluorescein isothiocyanate (FITC)-labeled secondary antibody. The controls were labeled with the secondary antibody alone. Twenty thousand events were analyzed for each sample on a BD FACSort instrument using CellQuest software (San Diego, CA, USA).

Western-blot Assay
Cell lysates of Heparinase and LMWH treatment and the control groups were harvested. The separated proteins were detected by immunoblotting. Cell pellets were resuspended in PAGE sample buffer containing 1% SDS, and the samples were heated to 100°C for 3 min after centrifugation and transferred onto the nitrocellulose membrane. The membrane was blocked with 5% milk in TBST (20 mM Tris–HCl pH7.5, 0.5M NaCl, 0.5% Tween 20) at 37°C for 1 h, and then incubated with the monoclonal antibody to RSVF (Chemicon, Temecula, CA, USA) at 4°C overnight. After three washes with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), the cells were incubated with the horseradish peroxidase(HPR)-conjugated goat anti-mouse IgG for 2 h. After adding the HRP substrates, the dried membrane was exposed to Kodak X-omat autoradiography film and developed in a standard film developer. After the run, the acrylamide gel was placed in a transfer apparatus with a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA), and conditions for transfer were according to the manufacturer’s protocol.

Statistical Analysis
Data were presented as means±SE. After the homogeneity of variance, differences between groups were determined by the Bonferroni or Dunnett T3 test. Differences with \( p \leq 0.05 \) were considered significant. Statistical analysis was carried out with Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA, version 11.0).

Results

RSV Infection of Different cell Lines in vitro
Cells were inoculated with RSV and incubated at 37°C for 24h. Hela, HEK-293 and Hep-2 cells were RSV-positive, while Lo2 was unsusceptive to RSV infection. The percentage of infected cells for each cell line was determined by flow cytometry (Figures 1, 2A).

RSV Infection of Different cell Lines Pretreated with Heparinase and LMWH
The infection of RSV to Hela cells pretreated with Heparinase and LMWH was same as that of the untreated group (Figure 2B), and the percents of RSV infection for HEK-293 cells were RSV-positive, while Lo2 was unsusceptive to RSV infection. The percentage of infected cells for each cell line was determined by flow cytometry (Figures 1, 2A).

Discussion
RSV is a major cause of respiratory morbidity, resulting in hospitalization for bronchiolitis in
some infected infants that is associated with wheeze in later life. However, there are a few reports about the susceptible hosts of RSV. It is realized the human lung is the susceptible host of RSV, and infection by RSV can cause extensive inflammation and lung damage. Otherwise, we have confirmed that RSV can induce nephropathy in rat model. In this study the susceptibility of four cell lines for RSV were detected, which were considered the good systems for in vitro analysis of lung, kidney and liver tissues response to RSV infection and virus-host interactions. As the positive control, the human cervix carcinoma cell line Hela was also used. Flow cytometry for RSVF was used to provide the basis for the analysis of successfully RSV infection, and western-blot was also applied for the detection of the expression of RSVF. In this protocol the human lung epithelial tumor cell line Hep-2 and human cervix carcinoma cell line Hela are highly susceptible to RSV infection. Human embryonic kidney cell line HEK-293 is also RSV-susceptible, while human liver cell line Lo2 is nearly RSV-negative. The experimental results present evidence that Hela, Hep-2 and HEK-293 are important reservoirs of RSV while Lo2 are not, which indicated the lung and the kidney but not the liver might be the susceptible hosts of RSV. It will now be very important to determine the phenotype of different cells as well as the receptors that lead to the tropism variation.

GAGs are linear, unbranched polymers of repeating disaccharide units produced by and associated with most mammalian cells and some bacterial cells. GAGs are found in intracellular vesicles and on the outer face of the plasma membrane, where they can act as virus receptors. Many studies have shown that some bacterias
The role of HS N-sulfation and iduronic acids

and viruses use GAGs, particularly HS, a member of GAGs, for attachment to, and entry of cultured, immortalized cells\textsuperscript{11-14}. We and others have established the dependence of respiratory syncytial virus (RSV) on HS for efficient attachment to and infection of cultured, immortalized cells\textsuperscript{7,8,15,16}. HS was synthesized by a non-sulfated precursor structure composed of alternating GlcUA and N-acetylated GlcN (GlcNAc) units. This precursor was modified through a series of enzymatic reactions, initiated by N-deacetylation and N-sulfation of Glc-NAc residues\textsuperscript{17,18}. As the unequal tissue origin, developmental and pathophysiological status, the length, N-sulfated group distribution and modification of HS chain were heterogeneous, and HS had different functions\textsuperscript{18-20}. In this study we also examined the effects of removal of HS chains from the cell surface on viral infection of these cells. Commercially, there are several enzymes that can cleave N-sulfation and iduronic acids units of HS, such as Heparinase I, II, III and LMWH. These enzymes were used to further investigate the role of N-sulfation and iduronic acids units of HS in RSV infection. After pretreating the cells with Heparinase I the iduronic acids units of HS reduced, and heparinase II led to lower N-sulfation and iduronic acids units, while heparinase III removed the N-sulfation units. As an analog of HS, LMWH may inhibit positively charged RSV infection through cooperative electrostatic association\textsuperscript{6}. All enzy-

Figure 2. \textbf{A}, The percentage of RSV-infected Hela, HEK-293, Hep-2 and Lo2 cells. \textbf{B}, \textbf{C} and \textbf{D}, The percentage of RSV-infected Hela(B), HEK-293(C) and Hep-2(D) cells pretreated with Heparinase I, II, III and LMWH.

Figure 3. The expression of RSVF in RSV-infected Hela (\textbf{A}), HEK-293 (\textbf{B}) and Hep-2 (\textbf{C}) cells pretreated with Heparinase I, II, III and LMWH and the control.
mantic treatments reduced the susceptibility of Hep-2 cells to RSV infection. After treatment with heparinase II and III HEK-293 was nearly negative to RSV infection, indicating that the N-sulfation of HS was important in RSV infection in HEK-293 cells. All enzymes could not reduce the susceptibility of Hela cells to RSV infection.

Conclusions

Thus, it will now be very important to determine the phenotype of different cells as well as the receptors that lead to the tropism variation.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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