Ability of low-molecular-weight heparin to alleviate proteinuria by inhibiting respiratory syncytial virus infection

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SUMMARY:

Aim: Low-molecular-weight heparin (LMWH) is a negatively charged glycoprotein and has a very similar structure to that of cell surface heparin sulfate (HS). Thus, LMWH, an analog of HS, may inhibit positively charged respiratory syncytial virus (RSV) infection through cooperative electrostatic association.

Methods: In this study, rats were respectively treated with 400 IU/kg LMWH before, during or after being inoculated with $6 \times 10^6$ plaque-forming unit (PFU) RSV. RSV and normal control groups were respectively inoculated by RSV and virus-free Dulbecco’s modified Eagle’s medium (DMEM). HeLa cells in vitro were pretreated with LMWH, elastase (ELA), heparinase (HpaIII) and protamine before being inoculated with $6 \times 10^1$ PFU RSV. RSV infectivity was determined by in situ hybridization and plaque assay.

Results: After inoculation, the urinary protein excretion and serum parameters in LMWH-treated rats were significantly lower than those in the RSV group. No abnormalities of glomerular structure were observed in LMWH-treated groups whereas swelling and slight hypercellularity in minority glomeruli and foot process effacement were observed in the RSV group. RSV RNA of LMWH-treated rats had weaker expression than that of the RSV group. In vitro, RSV infection in RSV + LMWH, HpaIII + ELAI, protamine + ELAI, ELAI, HpaIII and protamine treatment cells were significantly lower than that of the RSV control, and that in RSV + LMWH was the least. There were no significant differences in RSV infection between ELAI + LMWH and RSV control.

Conclusion: Our study confirmed that there is a correlation between RSV and proteinuria in rats. LMWH can alleviate proteinuria in rats through inhibiting RSV from binding with HS which plays an important role in the onset of RSV infection.

KEY WORDS: elastase, heparin sulfate, heparinase, low-molecular-weight heparin, proteinuria, respiratory syncytial virus.
and so on. It has been shown that urinary GAG level is increased and GAG content of glomerular basement membrane (GBM) is decreased in NS.17–25 A recent report by Birmelle et al.26,27 suggests that one or several factors might modify HS and anionic charge in glomerular epithelial cells and GBM, and thereby increase glomerular permeability to albumin. Low-molecular-weight heparin (LMWH) is a negatively charged glycoprotein due to N-sulfate and O-sulfate on the carbon chain and has a very similar structure to that of cell surface HS. Thus, LMWH is frequently used as a convenient analogue of HS for experimental purposes as in the present study. We presumed that RSV may bind to HS on glomerular surface through cooperative electrostatic association on account of negatively charged HS, and then penetrate into glomeruli to cause the disease. We describe a rat model treated with LMWH to study the effects of LMWH on proteinuria in rats being inoculated with RSV. Because HS is widespread and exists on most mammalian cell surfaces, we used HeLa cells in vitro pretreated with Hpa, ELA, LMWH and protamine (positive charge) to study the effects on RSV infection to further explore the interaction between RSV and cells.

METHODS

Virus and cells

Human RSV, long strain and HeLa cell line were obtained from the Viral Institute of the Chinese Academy of Preventive Medical Science. HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% foetal calf serum (FCS) at 37°C in 5% CO2; RSV was cultured in HeLa cells and assayed for infectivity as previously described.1

Animals

Male Sprague–Dawley rats, weighing 140–170 g and free of specific pathogens (West China Medical Animal Centre, Sichuan University, Chengdu, China), were divided into five groups at random. They were sequentially inoculated intranasally (0.2 mL) and i.p. (0.4 mL) with pathogens (West China Medical Animal Centre, Sichuan University, Chengdu, China), were divided into five groups at random. They were simultaneously inoculated intranasally (0.2 mL) and i.p. (0.4 mL) with 6 ¥ 10^6 plaque-forming units (PFU) RSV and treated i.p. with LMWH 400 IU/kg (Clivarin, 1432 anti-Xa I.U.; BASF Pharma, Knoll, Germany). In group A (n = 5), RSV was given in the first 3 days, and LMWH was given for the following 11 days to investigate if LMWH could alleviate or prevent the damage of glomeruli HS by RSV. In group B (n = 5), the mixture of RSV and LMWH was given in the first 3 days, and LMWH was given for the following 11 days to observe if LMWH could alleviate or prevent the damage of glomeruli HS by RSV. In group C (n = 5), LMWH was continuously given throughout the experiment and on days 4, 5 and 6 RSV was also given to explore if LMWH competing with HS of glomeruli to associate with RSV, could inhibit RSV attachment to HS. The RSV (n = 5) and normal control (n = 5) groups were respectively inoculated with RSV and virus-free DMEM for 3 days. The far, behaviour, appetite, respiration and weight of the rats were monitored.

Measurement of proteinuria and serum parameters

Twenty-four h urine collected by metabolic cage was used to measure proteinuria excretion (pyrogallol end-point method). The blood samples were harvested from the orbital veniplex of the rats on the 14th day after inoculation to measure serum albumin, cholesterol, urea nitrogen and creatinine (Hitachi 7600; Hitachi, Tokyo, Japan).

Histopathological studies

Kidney paraffin sections (4 µm) were stained with haematoxylin–eosin for routine morphological study. Fresh renal tissues were fixed in 3% glutaral for ultrastructural study under a H-600IV transmission electron microscope (Hitachi).

In situ hybridization of renal tissue

In situ hybridization was employed to compare RSV infection in renal tissues of all rats. RSV-specific probes composed of three oligonucleotides with sequences chosen from the published sequence of G protein mRNA of RSV (sequence I, 5′-CTTTTCTAGTGTCTTAGCGGT GCCTTGGTCTCTTG-3′; sequence II, 5′-GCAGGTTTGACCTG ACTCTCGTTGTTGGTAGACG-3′; sequence III, 5′-CTCTTCCT GTGGCTTGGTGTTGGTAGTCTCTTT-3′) were synthesized by Gene-base Biology (Shanghai, China). The oligonucleotides were end-labelled with digoxigenin and hybridization was carried out in a hybridization detection kit according to the manufacturer’s instruction (DakoCytoptation, Glostrup, Denmark) and with in situ hybridization used as reference.28 Kidney sections (4 µm) were deparaffinized with xylene, rehydrated through decreasing concentrations (from 100% to 70%) of ethanol, inactivated with endogeno enzymes by 3% hydrogen peroxide at room temperature for 5–10 min, and digested with 0.1 mg/mL proteinase K at 37°C for 10 min. Prehybridization solution was then added to the sections, which were kept at a temperature of 35–42°C for 2–4 h. Hybridization was carried out by treating with 20 µL hybridization solution containing 20–50 ng probes and keeping overnight in a wet cabinet supplemented with 20% glycerine at 35–42°C. On the following day, sections were rinsed successively twice in 2 x standard sodium citrate, once in 0.5 x standard sodium citrate, and once in 0.2 x standard sodium citrate for 15 min each, treated sequentially with confining liquid, biotinylated mouse anti-digoxin, streptavidin biotin complex and biotinylated peroxidase, coloured with 3,3′-diaminobenzidine tetrahydrochloride, and counterstained with haematoxylin. The slides containing RSV-infected HeLa cells served as positive controls and the sections treated without probes served as negative controls. The positive hybridization signal locating intracytoplasm presented as dark brown. The images were collected by an image analytical system (Nikon & Spot, Japan).

HeLa cell in vitro

HeLa cells were plated on the slides treated with polylysine in 12 well plates at 1 ¥ 10^5/well and maintained in DMEM containing 10% FCS at 37°C in 5% CO2. When HeLa cell monolayers were confluent, they were divided into nine groups according to various purposes: (i) RSV + LMWH, 6 ¥ 10^5 PFU RSV was mixed with 50 µg/mL LMWH, which was added into HeLa cells after 1 h; (ii) protamine, HeLa cells were incubated with 25 µg/mL protamine (Pharmaceutical Factory of Shanghai, Shanghai, China) for 1 h, then inoculated with 6 ¥ 10^5 PFU RSV; (iii) heparinase, HeLa cells were incubated with 25 µg/mL RSV for 1 h, then inoculated with 6 ¥ 10^5 PFU RSV; (iv) elastase, HeLa cells were incubated with 0.1 µg/mL ELA II for 1 h, then inoculated with 6 ¥ 10^5 PFU RSV; (v) heparinase + ELA, HeLa cells were incubated with 0.1 µg/mL ELA II for 1 h and subsequently with 0.1 µg/mL ELA I for 1 h, then inoculated with 6 ¥ 10^5 PFU RSV; (vi) protamine + ELA, HeLa cells were incubated
with 25 μg/mL protamine for 1 h and subsequently with 0.1 μg/mL ELAI for 1 h, then inoculated with 6×10⁴ PFU RSV; (vii) ELAI + LMWH, 0.1 μg/mL ELAI was mixed with 50 μg/mL LMWH for 1 h, which was incubated with HeLa cells for 1 h, then 6×10⁴ PFU RSV was inoculated; (viii) RSV, HeLa cells were incubated with phosphate-buffered saline (PBS) for 1 h, then inoculated with 6×10⁴ PFU RSV; and (ix) PBS, HeLa cells were only incubated with PBS. After a 1 h virus adsorption, the RSV was removed and cells were washed twice in D-Hanks and overlaid with DMEM containing 2% FCS for 2–3 days. In situ hybridization and plaque assay was used to determine RSV infectivity.

In situ hybridization

The slides were fixed in 4% paraformaldehyde containing 1% diethylpyrocarbonate for 20 min, washed in distilled water, treated with the mixture of 3% hydrogen peroxide and methanol (1:50) at room temperature for 30 min, and digested with pepsine containing 3% citric acid dilution at 37°C for 2 min. The following steps were the same as the hybridization of kidney sections described above. Five visual fields were selected randomly under the same condition and average integral optical density (IOD) of each visual field was calculated for statistics.

RSV plaque assay

HeLa cells in DMEM containing 10% FCS and 1% methylcellulose were fixed with 10% formalin at room temperature for 10 min and stained with 0.5% crystal violet for 10 min, and the plaques were enumerated under an inverted microscope. 

$$PFU = a \times b \times v$$

(a: means of plaques; b: multiple of virus dilution; v: milliliter of virus inoculum). RSV titre was expressed as log10 PFU/cells.

Statistical analysis

Statistical analysis was carried out with the Statistical Package for the Social Sciences ver. 11.0 (SPSS Software, Chicago, IL, USA). After the test of homogeneity of variance, the Bonferroni or Dunnett T3 test was used for group comparison. $P < 0.05$ was considered significant.

RESULTS

Proteinuria excretion

Compared with proteinuria excretion in the RSV group (32.041 ± 3.844 mg/24 h), there was no significant increase of that in groups A (7.405 ± 4.057 mg/24 h), B (7.101 ± 1.833 mg/24 h) or C (9.209 ± 1.625 mg/24 h) treated with LMWH ($P < 0.05$). Moreover, no significant differences were found between proteinuria excretion of every two LMWH-treated groups and compared with that in the normal control group ($P > 0.05$; Table 1).

Serum parameters

There was a decrease in serum albumin (15.060 ± 1.335 g/L) and an increase in blood urea nitrogen (12.920 ± 3.932 mmol/L) in the RSV group compared with those in the three LMWH-treated groups, but no statistical differences in creatinine and cholesterol in any of the groups were detected ($P > 0.05$; Table 1).

Histopathological findings

Light microscope

There were no abnormalities of the glomeruli but slight inflammatory cell infiltration in the interstitium detected in all of the LMWH-treated groups, while slight hypercellularity in minority glomeruli, swelling and vacuolar degeneration in partial tubular epithelial cells, together with slight inflammatory cell infiltration in the interstitium, were observed in the RSV group (Fig. 2).

Electron microscopy

Glomerular structures of the LMWH-treated groups were almost normal, and no swelling and separation of foot process and GBM were apparent. Nevertheless, effacement of foot process of glomerular epithelial cells was exhibited in the RSV group. No electron-dense deposit was found in any of the groups (Fig. 3).

In situ hybridization

The dark-brown grains indicating positive hybridization signals appeared in the epithelial cells and mesangial cells of glomeruli and tubular epithelial cells. Nevertheless, the signals of groups A, B and C were weaker than those seen in the RSV group. No positive signal was detected in the control (Fig. 4).

Analysis of infectivity of RSV

In situ hybridization

In RSV + LMWH, HpaIII + ELAI, protamine + ELAI, ELAI, HpaIII and protamine, the positive signals of RSV
RNA in cytoplasm expressed less than those in the RSV control group \((P < 0.05)\), and those in RSV + LMWH were the least \((P < 0.05)\). There were no significant differences in \(\text{in situ} \) hybridization among HpaIII + ELAI, protamine + ELAI, ELAI, HpaIII and protamine \((P > 0.05)\). The positive signals of RSV RNA in cytoplasm were not significantly different between ELAI + LMWH and the RSV control \((P > 0.05)\), whereas ELAI + LMWH showed more positive signals than those in RSV + LMWH, HpaIII + ELAI, protamine + ELAI, ELAI, HpaIII and protamine \((P < 0.05; \text{Table 2})\).

**Plaque assay**

Table 2 illustrates that the PFU in RSV + LMWH, HpaIII + ELAI, protamine + ELAI, ELAI, HpaIII and protamine...
Fig. 3 Ultrastructure of the renal tissues of rats under an electron microscope. Normal and clear foot processes were presented in group A (3a; original magnification, ×5000), B (3b; original magnification, ×10 000) and C (3c; original magnification, ×10 000). Foot process effacement was extensive in the respiratory syncytial virus group (3d; original magnification, ×17 000).
were lower than those in the RSV control (P < 0.05), and those in RSV + LMWH were the least (P < 0.05). The PFU in protamine were more than those in RSV + LMWH, HpaIII + ELAI, protamine + ELAI, ELAI and HpaIII (P < 0.05), while there were no significant differences among those in HpaIII + ELAI, protamine + ELAI, ELAI or HpaIII (P > 0.05). There were no significant differences of the PFU between ELAI + LMWH and the RSV control (P > 0.05). After treatment with 0.5 μg/mL ELAI, the exfoliation of cells appeared.

Table 2  Analysis of integral optical density in situ hybridization and respiratory syncytial virus plaque assay

<table>
<thead>
<tr>
<th>Groups</th>
<th>IOD (x10³)</th>
<th>Plaque-forming unit</th>
<th>RSV infectivity (%RSV control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>① RSV + LMWH</td>
<td>10.51 ± 3.35*†</td>
<td>12.00 ± 5.57</td>
<td>22.62 ± 3.24*†</td>
</tr>
<tr>
<td>② Protamine</td>
<td>42.01 ± 8.30*</td>
<td>32.00 ± 3.61</td>
<td>62.04 ± 11.22*</td>
</tr>
<tr>
<td>③ HpaIII</td>
<td>39.82 ± 4.62*</td>
<td>21.33 ± 1.53</td>
<td>41.23 ± 4.78*</td>
</tr>
<tr>
<td>④ ELAI</td>
<td>34.13 ± 8.19*</td>
<td>23.67 ± 5.51</td>
<td>46.20 ± 4.31*</td>
</tr>
<tr>
<td>⑥ HpaIII + ELAI</td>
<td>32.57 ± 8.09*</td>
<td>20.33 ± 1.53</td>
<td>39.36 ± 5.62*</td>
</tr>
<tr>
<td>⑦ Protamine + ELAI</td>
<td>32.98 ± 5.64*</td>
<td>18.67 ± 1.15</td>
<td>35.94 ± 1.46*</td>
</tr>
<tr>
<td>⑧ RSV + LMWH</td>
<td>60.87 ± 14.21Δ</td>
<td>46.33 ± 3.51</td>
<td>89.80 ± 13.74Δ</td>
</tr>
<tr>
<td>⑨ RSV</td>
<td>67.79 ± 16.40</td>
<td>52.00 ± 4.00</td>
<td>100</td>
</tr>
<tr>
<td>⑩ PBS</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are presented as means ± standard deviation (SD). *P < 0.05 vs RSV control; †P < 0.05 vs ①–⑥; P < 0.05 vs ① & ③–⑥. ELA, elastase; IOD, integral optical density; LMWH, low-molecular-weight heparin; PBS, phosphate-buffered saline; RSV, respiratory syncytial virus.
DISCUSSION

This study represented a follow up from our research group. In vivo: (i) after RSV inoculation, proteinuria and the histopathological changes in rats were significantly alleviated with LMWH treatment; (ii) the mixture of RSV and LMWH was given, which did not cause severe proteinuria and histopathological changes in rats; and (iii) LMWH was given first, and then RSV was inoculated, which prevented proteinuria and the histopathological changes in rats. The results indicated that LMWH could inhibit RSV from damaging glomeruli. As LMWH has a very similar structure to that of GBM HS, we presume that two mechanisms in the initial stage of RSV nephropathy might play key roles: (i) positively charged RSV binds to negatively charged LMWH through cooperative electrostatic association, which prevents RSV attachment to HS in the GBM and thus keeps the anionic layer in the GBM from being damaged; (ii) as an exogenous analogue of HS, LMWH competes with endogenous HS in binding with RSV, which lowers the viral infectivity.

Although the pathogenesis of MCNS is not entirely clear, the alteration of the charge-selective function of the GBM is considered to be one of the main causes of the proteinuria in NS. GAG form the main components of anionic sites of the GBM, and HS side-chain is the chief GAG in the GBM. Decreased GAG levels in the GBM have been documented in human NS and in animal models. Decreased HS content of GBM in NS results in loss of negative charge from the GBM, which may explain the increased passage of proteins into the urine. Therefore, we hypothesized that cationic charge of RSV may attach to anionic HS on glomerular surface through electrostatic association, which causes the damage of glomeruli and increasing permeability of GBM in the initial stage. It is evident that this process can be stopped by LMWH.

In our early study, the damage of RSV to rat kidney occurred in two stages. In the first stage, the damage to the kidney was directly caused by RSV in rats, which confirmed that RSV was being caused by RSV in rats, which confirmed that RSV was capable to damage the kidney in rats. After RSV was neutralized by LMWH, the proteinuria was significantly alleviated, which also indicated that the interaction between RSV and GBM HS possibly worked.

Furthermore, our study explored the interaction between RSV and cells in vitro. HS generally locates on most mammalian cell surfaces, and HeLa (human cervical carcinoma cell line) and HEP2 (human epithelial cell line) and so forth are commonly used for virus multiplication and isolation, so we selected HeLa cells. The results showed:

1. Evidence that the infectivity of RSV obviously fell to 22.62% after incubating with LMWH (HS), further supporting the former argument, and cation of RSV was neutralized by anion of LMWH which might stop its binding with HS to lower RSV infection, which was confirmed by the results in vivo as well.

2. Protamine, charged positively, is able to bind anionic molecules on cell surface, such as HS, by static electricity, which destroys the anionic layer of the cell membrane. The infectivity of positively charged RSV decreased to 62.04% in protamine pretreatment cells, suggesting that RSV infect cells by binding to HS on the cellular surface with cooperative electrostatic association.

3. HpaIII, the unique internally tangent glucuronidase in mammal, specifically degrades HS. It can cleave and degrade HS by destroying specific binding sites between the core protein and HS chain, cleaving within S-domains which are IdoUA, N- and O-sulfate group-rich sequences and highly modified and/or mixed sequences where modified and unmodified disaccharides alternate, which removes the HS from the cell surface. The infectivity of RSV in HpaIII pretreatment cells reduced to 41.23%, suggesting that there is a decrease of RSV infection when HS was degraded by HpaIII, which led negative electrons to be reduced to prevent RSV invade HeLa cells, and efficient RSV infection to cells requires the HS on the cell surface.

Yet, the sequence of RSV infectivity of the three groups was RSV + LMWH to HpaIII to protamine, suggesting that the role of cell surface HS in RSV infection is not based on a simple charge interaction between the virus and sulfate groups but still involves another specificity.

What is more, our study showed that:

1. ELA can degrade heparin sulfate proteoglycan on the cell surface to lose HS, and its cation also can neutralize anion of HS, which may reduce the binding between RSV and HS to lower RSV infection. RSV infection in cells being treated with ELA was decreased, but that of ones being treated with HpaIII + ELA, HS on cellular surface was resolved by HpaIII, which lowered RSV infection and led ELA not to interact with HS, showed no statistical difference compared with that of ones being treated with ELA or HpaIII alone.

2. RSV infection in cells being treated with ELAI + LMWH was not significantly lessened, indicating that positively charged ELAI being neutralized by negatively charged LMWH lost its neutralization and enzymolysis on HS, after which HS on the cell surface might be completely bound with RSV, so there was no influence on RSV infection.

3. RSV infection in cells being treated with protamine + ELAI (35.94%) was significantly lower than that of ones being treated with protamine alone, indicating that negatively charged HS on the cellular surface was first neutralized with positively charged protamine, and then resolved by ELAI, which might lead anion of HS to be further diminished, and also suggests that the effect of ELAI on cell surface HS is not only based on a simple charge interaction.

4. A low dose of ELAI (0.1 µg/mL) could remove HS from the cell surface by degrading it to lessen anion on the cell surface. RSV infection was then inhibited...
because the virus could not efficiently bind to the cell surface.

5. HeLa cells was pretreated by a large dose of ELAII (0.5 μg/mL) and subsequently inoculated with RSV, after which the cells were obviously exuive, suggesting that the large dose of ELA may not just play a role in cell surface HS, but may also act on other components of the extracellular matrix to destroy the cellular membrane or disrupt cells and thus aggravate RSV infection.

Our study reveals for the first time that there is a correlation between RSV and the minimal change nephropathy-like syndrome in rats. Nevertheless, how RSV infection evokes developing glomerular damage still needs to be further explored. However, our study has provided strong evidence for the fact that cell surface HS plays an important role in the first step of RSV infection, associated with inflammatory enzymes. LMWH can prevent or reduce RSV infection in vivo and in vitro, which partly explains the clinical effect of heparin in NS and suggests that LMWH may be used to inhibit viral infection.

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