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The effect of hyaluronan on airway mucus transport and airway epithelial barrier integrity: Potential application to the cytoprotection of airway tissue

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ABSTRACT

The lubricating abilities and the protective functions of hyaluronan, a structural component of interstitial and connective tissues, were assessed in *in vitro* models of airway mucus transport and epithelial barrier. We found that hyaluronan enhanced the transport of airway mucus by cilia and by cough: the lower the hyaluronan molecular weight, the higher the increase. By immunofluorescence and western blot, we observed a significant dose-dependent (0.1, 1, 5 and 10 mg/ml) increase by low molecular weight hyaluronan (40 kDa) in the expression of tight junction proteins such as ZO-1, as well as an increase in the trans-epithelial resistance. Incubation of airway epithelial cells with hyaluronan 40 kDa also significantly increased the gap junction functionality. Finally, we demonstrated that hyaluronan 40 kDa protects the airway epithelium against injury induced by bacterial products during infection. These results demonstrate that the expression and functionality of intercellular adhesion molecules are increased by hyaluronan which can also act as a lubricant at the airway epithelium surface and suggest that hyaluronan may play a therapeutic role in a variety of respiratory diseases.

1. Introduction

The epithelial lining of the airway surface provides an efficient barrier against microorganisms and aggressive molecules (pollutants) through interdependent functions including mechanical clearance of the mucus, homeostasis of ion and water transport, biochemical anti-bacterial, anti-oxidant and anti-protease functions and a cellular barrier function by means of intercellular junctions. Efficient airway mucus transport either by ciliary activity or by cough is dependent on optimal airway epithelium/mucus interaction which could be improved by lubricant molecules.

Hyaluronan is described as one candidate molecule (Prestwich and Kuo, 2008). Hyaluronan is a large glycosaminoglycan that is abundant in the extracellular matrix of many tissues and is involved in vertebrate tissue morphogenesis and cellular processes (Noble, 2002; Spicer and Tien, 2004; Rosines et al., 2007; Garcia-Fuentes et al., 2009; Rodriguez et al., 2011). In the airways, it is an important component of the extracellular matrix in the lung parenchyma and plays a key role during lung development (Calvitti et al., 2004). Data in the literature showing that hyaluronan accumulates to high levels at the margins of wounds (Oksala et al., 1995) and in wound fluids (Longaker et al., 1991) suggest that hyaluronan may also play a key role in wound healing, which is an important process in the restoration of the epithelial barrier integrity.

After injury, the barrier function will re-establish after several days but relative leakiness will correspond to a “low protective” barrier against successive aggressive agents. Therapy of airway epithelium injury and repair should aim to improve the cytoprotection of the airway epithelium against injury. Venge et al. (1996) verify the hypothesis that hyaluronan administration subcutaneously may reduce the number of bacterial infections in patients with chronic bronchitis. They conclude that hyaluronan may act possibly by enhancing cellular host defense mechanisms. From the perspective of published work thus far, there is evidence that hyaluronan may exert a protective effect against injury in a number of respiratory diseases (Turino and Cantor, 2003). However few details of the mode of action are available.

The aim of the present work was therefore to study the effect of hyaluronan of different molecular weight, obtained from microbial fermentation, on airway mucus transport and on the expression and functionality of intercellular adhesion molecules involved in the airway epithelial barrier integrity. In addition, we analysed the protective effect of hyaluronan against the virulence factors produced by *Staphylococcus aureus*, a bacterium frequently involved in pulmonary infections.

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2. Results

2.1. Hyaluronan enhances airway mucus transport

Effective cilia-mucus interaction is necessary for an efficient mucus transport by cilia and by cough. Hyaluronan could restore an altered interaction. Reconstituted airway mucus was tested for its transport by ciliary activity and cough mechanism in presence of hyaluronan solutions at different molecular weights deposited at the interface between the *in vitro* airway epithelium or the simulated trachea and the mucus.

As shown in Fig. 1, compared to native mucus, the presence of hyaluronan significantly enhanced the mucus transport by cough. This increase was dependent on the hyaluronan molecular weight: the lower the molecular weight, the higher the increase of cough transport. Compared to the effect of water, the effects of hyaluronan 80 kDA and hyaluronan 40 kDa were significantly higher ($p<0.005$ and $p<0.0001$, respectively).

Fig. 2 shows the effect of hyaluronan solutions on mucus transport by ciliary activity. The low mucociliary transport rate observed with native mucus was significantly enhanced by water ($p<0.005$) and by hyaluronan solutions ($p<0.005$ or $p<0.001$), with a higher increase in presence of hyaluronan at low molecular weight. Whatever the molecular weight, the effect of hyaluronan was significantly higher compared to the effect of water ($p<0.05$ for hyaluronan 1000 kDa and $p<0.005$ for hyaluronan 80 kDa and hyaluronan 40 kDa).

Fig. 3 displays pictures of airway mucus droplets embedded or not with water or hyaluronan 40 kDa at 1 mg/ml. The mean contact angle, measured from these pictures, was $40.5 \pm 16.7°$ for the native airway mucus samples. When embedded with hyaluronan at 1 mg/ml or water, the contact angle of the airway mucus significantly dropped down to $29.6 \pm 13.3°$ for hyaluronan 40 kDa, $26.3 \pm 12.6°$ for hyaluronan 80 kDa, $28.6 \pm 13.1°$ for hyaluronan 1000 kDa ($p<0.005$) or to $30.5 \pm 11.6°$ ($p<0.05$) for water. Whatever the molecular weight, the effect of hyaluronan was not significantly different compared to the effect of water.

2.2. Hyaluronan enhances tight junction protein expression and functionality

Tight junctions play a key role in the protection of the airway epithelium by preventing the pericellular passage of noxious agents. The second step of our work was to evaluate the role of hyaluronan on airway epithelium tightness and integrity. First, airway epithelial cells were incubated with 1 mg/ml of hyaluronan solutions at different molecular weight all along the cell culture. Fig. 4 displays the effect of hyaluronan molecular weight on trans-epithelial resistance (TER)

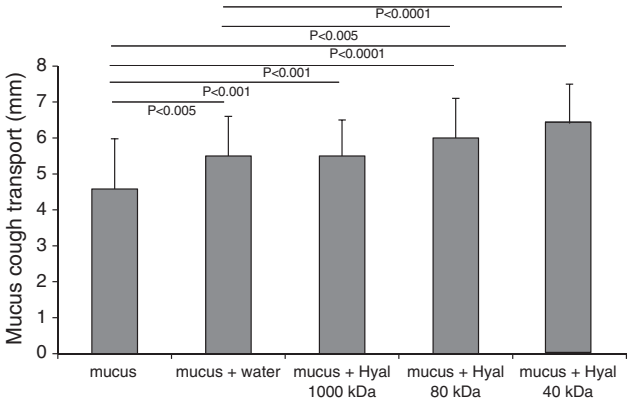

Fig. 1. Effect of airway mucus embedding by water or hyaluronan on mucus cough transport. Data are expressed as mean ± standard deviation for n = 12 samples.

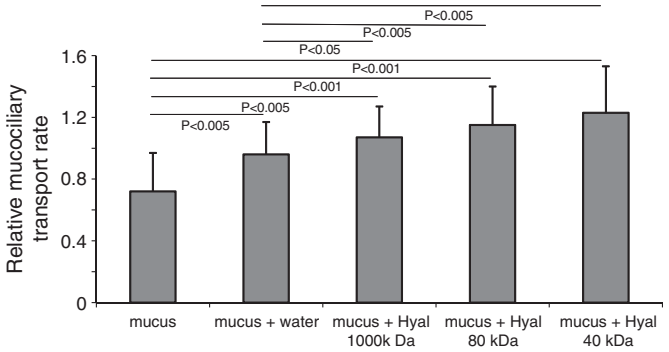

Fig. 2. Effect of airway mucus embedding by water or hyaluronan on mucociliary transport rate. Data are expressed as mean ± standard deviation for n = 12 samples.

measured 48 h after cell confluence was reached: the lower the molecular weight, the higher the TER. The increase in trans-epithelial resistance was significant for hyaluronan 80 kDa ($p<0.01$) and hyaluronan 40 kDa ($p<0.005$) compared to control. The effect of hyaluronan 40 kDa was significantly higher ($p<0.01$) compared to the effect of hyaluronan 80 kDa.

Then airway epithelial cells were incubated with increasing concentrations of low molecular weight hyaluronan (40 kDa, this molecular weight was selected for all the following experiments) and TER was measured daily. As shown in Fig. 5, the incubation of airway cells with hyaluronan 40 kDa during cell culture significantly enhanced the TER in a dose- ($p<0.01$) and time- ($p<0.01$) dependent way. This increase in TER can be related to the increased expression of the tight junction protein ZO-1, as shown in Fig. 6. By immunofluorescence, we observed that in the control cell culture, ZO-1 formed a relatively weak network (Fig. 6A) whereas in the presence of hyaluronan 40 kDa the network formed by ZO-1 was considerably denser (Fig. 6B, in the presence of 5 mg/ml of hyaluronan 40 kDa, 48 h after confluence). These data are confirmed by western blot as demonstrated in Fig. 6C where ZO-1 expression is shown to be significantly increased ($p<0.05$) when the cells were incubated with 5 or 10 mg/ml of hyaluronan 40 kDa.

2.3. Hyaluronan protects airway epithelial cells from death induced by bacterial virulence factors

During infection, bacteria secrete virulence factors that may injure the airway epithelium. To investigate the protective effect of hyaluronan, airway epithelial cells were continuously treated during culture with 5 mg/ml or 10 mg/ml of hyaluronan 40 kDa. 48 h after confluence, the cells were incubated for 24 h with 0.2%, 2% or 20% of *S. aureus* supernatant or Trypticase Soy Broth (TSB) and DNA fluorescent probes. The cell death index, measured after 24 h of incubation with different concentrations of *S. aureus* supernatant, is shown in Fig. 7. Interestingly, even in the absence of *S. aureus* supernatant, the cell death index was significantly lower ($p<0.05$) when the cells were incubated with 10 mg/ml of hyaluronan 40 kDa. The cell death index significantly increased ($p<0.01$) with increasing concentrations of *S. aureus* supernatant, and this effect was significantly ($p<0.01$) reduced in the presence of 0.2% or 2% of *S. aureus* supernatant when the cell were pre-incubated with 5 or 10 mg/ml of hyaluronan. It is noteworthy that even when the cells were pre-treated with hyaluronan, their incubation with 20% *S. aureus* supernatant induced a dramatic increase in cell death.

2.4. Hyaluronan prevents the decrease of tight and gap junction functionality

TER, which is related to the presence of tight junctions between adjacent cells, was measured after 24 h of incubation with *S. aureus*