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Pleural mesothelium lubrication after hyaluronidase, neuraminidase or pronase treatment

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ABSTRACT

Coefficient of kinetic friction (μ) of pleural mesothelium has been found to increase markedly after mesothelial blotting and rewetting. This increase disappeared after addition of a solution with hyaluronan or sialomucin, though previous morphological studies showed that only sialomucin occurs in mesothelial glycocalyx. In this research we investigated whether μ of rabbit pleural mesothelium increased after hyaluronidase, neuraminidase or pronase treatment. Hyaluronidase and neuraminidase did not increase μ, though neuraminidase cleaved sialic acid from mesothelial glycocalyx of diaphragm specimens, and removed hystochemical stain of sialic acid from glycocalyx. Sialomucin treated with neuraminidase lowered μ of blotted mesothelium, though less than untreated sialomucin; this feature plus lubrication provided by other molecules could explain why μ did not increase after neuraminidase. Short pronase treatment (in order to affect only glycocalyx proteins) increased μ; this increase was removed by hyaluronan or sialomucin. After pronase treatment μ decreased with increase in sliding velocity, indicating a regime of mixed lubrication, as in blotted mesothelium.

1. Introduction

Wang (1974, 1985) and Ohtsuka et al. (1997) by transmission electron microscopy found that colloidal iron stain of mesothelial glycocalyx persists after hyaluronidase treatment, but is removed by neuraminidase treatment, which cleaves sialic acid from sialomucin. In line with the earlier proposal of Andrews and Porter (1973), that the polyanionic nature of the surface of the mesothelial glycocalyx may protect from friction, Ohtsuka et al. (1997) concluded that the negative charges of sialomucin produce repulsive forces between facing serosal surfaces, and may, therefore reduce friction. Later on, D'Angelo et al. (2004) measured the coefficient of kinetics friction (μ) between specimens of rabbit visceral and parietal pleura, during oscillatory sliding in vitro at physiological velocities and loads. With Ringer bicarbonate between the mesothelia μ was 0.027, and did not change with changes in sliding velocity, consistent with boundary lubrication. Moreover, they found that μ increased markedly after having blotted the mesothelial surface with filter paper for 1–2 min, and that this increase was only reduced partially by rewetting the blotted mesothelium with Ringer solution. More recently, with the experimental approach of D'Angelo et al. (2004), we showed that addition of sialomucin (25 mg/ml) or hyaluronan (2.5 mg/ml) in Ringer after a standard blotting of the mesothelium brought μ back to its pre-blotting value (Bodega et al., 2012). The same effect was obtained by the addition of a mixture of sialomucin (12.5 mg/ml) and hyaluronan (1.25 mg/ml) in Ringer. Moreover, we found that, after washout of the solution with these macromolecules, μ with Ringer increased, without reaching its preceding post-blotting value. Finally, transmission electron micrographs of pleural specimens after mesothelial blotting showed that microvilli were partially or largely removed from the mesothelium, consistent with a substantial loss of the macromolecules normally entrapped among them (Bodega et al., 2012). Hence, our findings showed that hyaluronan and sialomucin are able to restore good lubrication in damaged mesothelium, and suggested that sialomucin may be involved in mesothelial lubrication under physiological conditions, while this should not be the case for hyaluronan because of the morphological findings of Wang (1974, 1985), and Ohtsuka et al. (1997). It could, therefore, be interesting to test whether μ of pleural mesothelium does not change after hyaluronidase treatment, but increases after neuraminidase treatment. The use of the latter enzyme, however, may be deceptive for various reasons. First, sialic acid (or neuraminic acid), which is placed at the outermost end of the sugar chain of glycoproteins (like sialomucin), is the precursor of several 9-carbon acid sugars in which structural diversities are generated by various substitutions at the 4–9 carbon (Schauer, 1982; Varki and Diaz, 1983; Varki, 1992, 1997). Some of these substitutions can markedly slow or even prevent the release of sialic acid by commonly used neuraminidases (Varki, 1992).

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The neuraminidase from *Streptococcus sanguis* has been found to cleave several isomers of sialic acid (Varki and Diaz, 1983), but this enzyme is not commercially available. Moreover, commonly used neuraminidases have a peak activity at pH 5–6, and therefore, their activity at neutral pH may be markedly reduced. Hence, the results obtained by treating the mesothelial specimens with neuraminidase should be taken cautiously. Finally, it could be interesting to test whether μ of the pleural mesothelium increases after treatment of the specimens with a protease for a short time, so as to digest only the proteins of the glycocalyx surface without altering the mesothelial cells.

The purposes of the present research are, therefore, the following. (1) To test whether μ of pleural mesothelium does not change after hyaluronidase treatment of rabbit pleural specimens. (2) To test whether μ of pleural mesothelium increases after treatment of the specimens with a commonly used neuraminidase. Moreover, to determine whether sialic acid is cleaved by this enzyme from pleural specimens (like those used to measure μ), and from commercial sialomucin (as it should do). These experiments should provide some information that may help to unravel the complex matter mentioned above. (3) To test whether μ of pleural mesothelium increases after a short treatment of the specimens with pronase, a broad spectrum protease, that has been used to digest the glycocalyx of mouse (Simionescu et al., 1981, 1985) and frog capillary endothelium (Adamson, 1990).

2. Methods

Rib cage, lung, and diaphragm were obtained from 38 rabbits (2.6–3.5 kg b.w.) purchased from "G. Bettinardi", Momo (Novara). Animal experimentation was authorized by the Ministry of Health by decree N. 60/03A issued according to Order of the Excutive 116/92, in compliance with Directive 86/609/EC. Rabbits were anesthetized with an intravenous injection of 2 ml/kg of a mixture of pentobarbital sodium (Sigma, 12 mg/ml) and urethane (Sigma, 150 mg/ml). They were then heparinised (0.1 mg/kg) and killed by exsanguinations. After removal of the skin and superficial muscles, the antero-lateral sides of the rib cage, the lungs (with closed trachea), and the diaphragm were removed, and kept at room temperature (21–28 °C) in Ringer bicarbonate (in mM: Na^+ 139, K^+ 5, Ca^{2+} 1.25, Mg^{2+} 0.75, Cl^- 119, HCO_3^- 29, D-glucose 5.6) through which 95% O_2 and 5% CO_2 was continuously bubbled.

The apparatus used to measure the frictional force was that described by D'Angelo et al. (2004). It consists of a sliding platform connected through unextensible threads to the core of a differential transformer, and of a balance arm held stationary at its fulcrum by a force transducer. Tissues specimens to be tested were fixed with the pleural surface facing upwards to the sliding platform, which was driven sinusoidally by an electric motor, and, with the pleural surface facing downwards, to a perspex rod attached to one end of the balance arm. The balance arm was held horizontal, and counterweights added to its other end enabled to change the normal force applied to the tissue from ~0.5 to ~8 g, corresponding to a pressure on the mesothelium from ~0.8 to ~12.9 cmH_2O. The frictional force on the direction of motion was measured by the force transducer. The coefficient of kinetic friction (μ) was computed as the slope of the relationship between the load and the frictional force. The values of frictional force used were those recorded in the central 40% of the excursion (Bodega et al., 2013). The specimen of rib cage was fixed to the sliding platform by a peripheral frame; alternatively, the specimen of diaphragm was pinned to a flat cork on top of the platform. The specimen of the lung was held over the end of the rod with an O-ring. The sliding velocity was 1.9 cm/s, except otherwise stated.

Measurements of μ were made at room temperature (21–28 °C) under the following sequence of conditions. (1) Control: Ringer bicarbonate or Krebs phosphate (in mM: Na^+ 140.3, K^+ 4.4, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 125.3, SO_4^{2-} 11.2, $H_2PO_4^-$ 4.4, D-glucose 6); (2) after enzyme treatment (see below); (3) after washout of the previous solution. In case that μ was increased by the enzyme, after its washout μ measurements were also made 5 min after addition of a solution with hyaluronan (2.5 mg/ml) or sialomucin (25 mg/ml) on treated mesothelium, and after washout of the solution. In case that μ was not significantly increased by the enzyme, in order to test its activity, the enzyme was preincubated with its target molecule, and μ measurements were made under this sequence of conditions. (1) Control condition; (2) after mesothelial blotting with filter paper, as previously described (Bodega et al., 2012); (3) after rewetting with Ringer bicarbonare or Krebs phosphate; (4) after addition of enzymatically treated target molecule; (5) after washout of the previous solution; (6) after addition of untreated target molecule; (7) after wash out of the previous solution.

2.1. Enzyme treatment

The following enzymes were used: hyaluronidase from *Streptomyces hyalurolyticus* (Sigma H1136), neuraminidase from *Clostridium perfrigens* (Sigma N2876), and pronase from *Streptomyces griseus* (Sigma P8811).

In the experiments with hyaluronidase or neuraminidase, after μ measurements under control conditions on the lung-diaphragm specimens, the cork with the diaphragm was placed in a 5% CO_2 – 95% air humidified incubator (Napco, model 5415) at 37 °C, covered by a thin layer of the enzyme solution, and the piston with the lung specimen was kept on it by mean of an adequate device, and, thus, was also immersed in the enzyme solution. After the incubation time (see below) the piston and the cork were brought back to the apparatus in order to measure μ. The treatment with pronase was much shorter and, therefore, it was performed at room temperature without removing the piston and the cork from the apparatus.

Hyaluronidase was used at a concentration of 30 U/ml in Ringer at pH 7.4 for 90 or 120 min (Knepper et al., 1984). The activity of the enzyme was tested by incubating hyaluronic acid (2.5 mg/ml) with the same concentration of hyaluronidase for 90 or 120 min at 37 °C. This solution was then applied to blotted mesothelium rewetted with Ringer, and its effect on μ compared with that of a solution of untreated hyaluronic acid (2.5 mg/ml).

Neuraminidase was used at a concentration of 5 U/ml for 60 or 90 min (see below). The activity of neuraminidase from *C. perfrigens* has a peak at pH 5.5, and decreases to ~40% at pH 7.0 and to ~25% at pH 7.4 (Cassidy et al., 1965). For this reason, in preliminary experiments we tested the effect of pH on μ of our pleural specimens, and found that after 90 min the lower value of pH at which μ did not significantly increase was 7.0. Therefore, μ measurements with neuraminidase were performed at pH 7.0, and Krebs phosphate was used because in the incubator it maintains pH at this value for a much longer time than Ringer bicarbonate. On the other hand, it is worth to point out that the same neuraminidase at pH 7.4 (2 U/ml for 30 min at 37 °C) has been found to cleave ~60% of the sialic acid from tracheal muscles of guinea pig and rat (Kai et al., 1992).

Another problem with the neuraminidase used is that, according to the manufacturer, it may have a small protease activity. Hence, we checked whether the neuraminidase used had protease activity. To this end, a 0.5% solution of FITC labelled casein was incubated with 5 or 10 U/ml of neuraminidase for 90 min. To measure the amount of digested protein, casein was precipitated by addition of trichloroacetic acid 0.6 N, and fluorescence intensity in the supernatant was measured with a Perkin-Elmer fluorescence spectrometer MPF4, using an excitation wavelength of 495 nm, and recording emission at 524 nm (Twining, 1984). Pronase was used