Mucus glycoproteins from ‘normal’ human tracheobronchial secretion

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Mucous secretions were collected from tracheas of patients undergoing minor surgery under general anaesthesia with tracheal intubation, and mucus glycoproteins were isolated by using isopycnic density-gradient centrifugation in CsCl/guanidinium chloride. ‘Whole’ mucins were excluded from a Sepharose CL-2B gel, whereas subunits obtained after reduction were included. Trypsin digestion of subunits afforded high- M_r glycopeptides (T-domains), which were further included in the gel. The latter fragments are heterogeneous and comprise two or three populations, as indicated by gel chromatography and ion-exchange h.p.l.c. Rate-zonal centrifugation showed that the ‘whole’ mucins are polydisperse in size, with a weight-average M_r of $(14-16) \times 10^6$. The macromolecules were observed by electron microscopy, as linear and apparently flexible thread-like structures. Subunits and T-domains had weight-average contour lengths of 490 nm and 160 nm respectively. It is concluded that mucus glycoproteins are present in secretions from the healthy lower respiratory tract. The ‘whole’ tracheal mucins are assembled from subunits, which in turn can be fragmented into high- M_r glycopeptides corresponding to the oligosaccharide domains typically found in mucus glycoproteins. The size and macromolecular architecture of the tracheal mucins is thus similar to that observed for mucins from human cervical mucus, chronic bronchitic sputum and pig stomach, providing yet another example of this general design of these macromolecules, i.e. subunits assembled end-to-end into very large linear and flexible macromolecules.

INTRODUCTION

A thin layer of mucus covers the surface of the large airways (Yoneda, 1976; Sturgess, 1977; Reid *et al.*, 1982). Several types of secretory cells may contribute to this protective coating, including the serous and mucous cells of the submucosal glands, as well as the goblet cells of the surface epithelium (Florey *et al.*, 1932; Jones, 1978; Jeffery, 1978). Airway mucus is normally removed, together with any detritus it contains, by ciliary action. The cilia waft mucus to the larynx, where it is swallowed, and it is thus difficult to collect mucus from the healthy lower respiratory tract. Most studies of the composition and properties of respiratory mucus in man have therefore been performed on sputum expectorated by patients with airway disease (see, e.g., Creeth *et al.*, 1977; Jenssen *et al.*, 1980; Carlstedt & Sheehan, 1984; Slayter *et al.*, 1984; Rose *et al.*, 1984; Mikkelsen *et al.*, 1985; Sheehan *et al.*, 1986; Marianne *et al.*, 1987). The macromolecules regarded as the major determinants of the rheological properties of mucus are the high- M_r glycoproteins referred to as the mucus glycoproteins or the mucins. In contrast, Bhaskar *et al.* (1985, 1986) propose that the major macromolecular constituent of mucus from the unstimulated healthy airways is proteoglycan. Only in samples obtained after stimulation by, for example, cholinergic agonists were significant amounts of glycoproteins detected.

To improve our understanding of normal airway secretions and to provide a reference point for studies on mucus obtained from patients with respiratory disorders, secretions from individuals with no clinical signs of airway disease have been studied. Mucus glycoproteins could be isolated from such samples and were characterized as to their size and macromolecular ‘architecture’.

EXPERIMENTAL

Materials

Guanidinium chloride (practical grade) was obtained from Riedel-de-Haen or Sigma. Stock solutions (approx. 8 M) were treated with charcoal before use. Phenyl-methanesulphonyl fluoride was from Sigma.

Analytical methods

Sialic acid was determined with an automated version (Lohmander *et al.*, 1980) of the method described by Jourdan *et al.* (1971), and hexose as described by Goa (1955).

Slot-blotting followed by staining with periodic acid-Schiff reagent (PAS) or Alcian Blue was performed as described in detail by Thornton *et al.* (1989). In short, samples of ‘whole’ mucins and subunits were blotted on to nitrocellulose membranes (0.20 μ m pore size) with a

Abbreviations used: PAS, periodic acid-Schiff reagent.

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Schleicher and Schnell Minifold II slot-blot apparatus (Anderman, Kingston upon Thames, U.K.). Membranes with a smaller pore size (0.025 μm) pretreated with poly-L-lysine (5 μg of poly-L-lysine in 50 μl of water was added to each well) were used for the T-domains, which were analysed after dialysing the individual column fractions in a multi-well dialysis apparatus (Gibco, Paisley, Scotland, U.K.). The blotted membranes were stained either with PAS or with Alcian Blue at pH 2.5, and the colour yield was measured with a microcomputer-based image-analysis system.

Collection of mucus samples

Mucus samples were obtained from 26 patients (age range 2–16 years) undergoing minor dental surgery under general anaesthesia with tracheal intubation. All were healthy non-smokers, with no clinical evidence of acute or chronic respiratory disease. The patients received no premedication, specifically no anticholinergic agents. Anaesthesia was induced with intravenous administration of thiopentone or by inhalation of halothane in O_2 , and was maintained with N_2O , O_2 and halothane with spontaneous respiration. Before extubation, secretions were aspirated with a catheter introduced into the trachea through the tracheal tube. Immediately after collection, each specimen was washed from the catheter and, if necessary, from the trap, with 5 ml of 6 M-guanidinium chloride/0.01 M-sodium phosphate buffer, pH 6.5, supplemented with 5 mM-*N*-ethylmaleimide and 0.1 mM-phenylmethanesulphonyl fluoride. Most specimens (21 of 26) were visibly free of blood.

Isolation of mucus glycoproteins

The samples were pooled into three lots (12 and 9 visibly free of blood; 5 with a slight contamination) and mixed with 100 ml of cold 6 M-guanidinium chloride/5 mM- Na_2EDTA / 5 mM-*N*-ethylmaleimide / 0.1 mM-phenylmethanesulphonyl fluoride/10 mM-sodium phosphate buffer, pH 6.5 and left stirring slowly overnight in the cold. After high-speed centrifugation (Beckman J2-21 centrifuge; JA-20 rotor; 18000 rev./min; 4 °C; 25 min) the pellet was resuspended in 25 ml of 6 M-guanidinium chloride/5 mM- Na_2EDTA /10 mM-sodium phosphate buffer, pH 6.5, by using a few gentle strokes with the loose pestle of a Dounce homogenizer, and was left with slow stirring in the cold for 24 h. After high-speed centrifugation (as above), the supernatants were pooled, CsCl and buffer were added to an initial density of approx. 1.40 g/ml in 4 M-guanidinium chloride, and isopycnic density-gradient centrifugation was performed in a Beckman L5-65B ultracentrifuge (50.2 Ti rotor; 90 h; 15 °C; 36000 rev./min). Fractions were collected from the bottom of the tubes and analysed for sialic acid, hexose, A_{280} and density. The last measurements were performed by using a Carlsberg pipette as a pycnometer. Fractions containing mucins were pooled and subjected to a second density-gradient-centrifugation step in CsCl/0.2 M-guanidinium chloride (initial density approx. 1.52 g/ml). Preparations of subunits were obtained after reduction of 'whole' mucins and T-domains after subsequent trypsin digestion (Carlstedt *et al.*, 1983b).

Gel chromatography

'Whole' mucins, subunit and T-domain preparations (approx. 100 μg in 0.5 ml) were chromatographed on a column (0.9 cm $\times$ 60 cm) of Sepharose CL-2B eluted with

4 M-guanidinium chloride, pH 7.4, at a flow rate of 2 ml/h. Fractions (0.5 ml) were analysed with PAS staining after slot-blotting on to nitrocellulose membranes. The void volume and the totally included volume were determined with Dextran Blue and *N*-acetylneuraminic acid respectively.

High-performance ion-exchange chromatography

T-domain preparations (approx. 200 μg) were chromatographed on a Pharmacia Mono Q HR 5/5 ion-exchange column using a 2150 LKB titanium-head pump connected to a 2152 LKB controller, a 2040-203 LKB mixing valve and a Pharmacia V-7 injector. Gradients were formed at the low-pressure side, and the column was eluted (0.5 ml/min) with 10 mM-piperazine perchlorate buffer, pH 5.0 (starting buffer), for 10 min followed by a linear gradient (60 min) to a final concentration of 0.25 M- LiClO_4 in starting buffer. Fractions (0.5 ml) were analysed with PAS and Alcian Blue after slot-blotting on to nitrocellulose membranes.

Rate-zonal centrifugation

This was performed on 'whole' mucins on a guanidinium chloride gradient as described by Sheehan & Carlstedt (1987). The sample (200 μl in 4 M-guanidinium chloride) was layered on to the preformed gradient with a Hamilton syringe and spun for 2.5 h at 40000 rev./min and 20 °C in a 6 $\times$ 4.2 Ti swing-out rotor (MSE). The tubes were unloaded from the top into 14 fractions (300 μl) directly into light-scattering cells, and the concentration of the macromolecules was estimated by PAS staining after slot-blotting on to nitrocellulose membranes, with mucins prepared from chronic-bronchitic sputum as a standard. The refractive index was measured on the individual fractions with an Abbé refractometer and was used to determine the guanidinium chloride concentration and thus the viscosity of the solutions.

Light-scattering

Quasi-elastic measurements on 'whole' mucins were performed on a Malvern 4700c system equipped with a Siemens 30 mW He/Ne laser. The *z*-average translational diffusion coefficient (D_t) was determined at a scattering angle (θ) of 20° and a temperature of 25 °C. Correlograms were constructed from the best of 10 separate runs, and the value of D_t was determined by using cumulant analysis with the standard Malvern software. Ten independent correlograms were obtained for each sample, and the mean value of D_t was corrected to 4 M-guanidinium chloride and 20 °C. Data were obtained for the unfractionated 'whole' mucins and from fractions 3–11 after rate-zonal centrifugation, the latter accounting for more than 90 % of the starting material. The radius of the equivalent hydrodynamic sphere (R_h) was determined from the Stokes–Einstein equation, and values of M_r were calculated from the equation $1/D_t = 10.5 \times 10^3 M_r^{0.53}$, derived by using data obtained previously for cervical mucins under identical conditions (Sheehan & Carlstedt, 1987).

Electron microscopy

Electron microscopy of 'whole' mucins and preparations of subunits and T-domains was performed as described by Sheehan *et al.* (1986). In short, the molecules were spread in a monolayer of benzyldimethyl-

Fig. 1. Isopycnic density-gradient centrifugation of human 'normal' tracheobronchial mucins in (a) CsCl/4 M-guanidinium chloride and (b) CsCl/0.2 M-guanidinium chloride

For full experimental details see the text. Fractions were analysed for sialic acid (Δ), hexose ($\bullet$), A_{280} ($\blacktriangle$) and density ($\blacksquare$) and pooled as indicated by the horizontal bars.

Fig. 2. Gel chromatography on Sepharose CL-2B of tracheobronchial mucins and fragments thereof

For full experimental details see the text. The fractions were analysed with the PAS reagent after slot-blotting on to nitrocellulose membranes. Key: —, whole mucins; ---, subunits; - · -, T-domains; V_0 , void volume; V_t , totally included volume.

Fig. 3. High-performance ion-exchange chromatography of T-domains on Mono Q

For full experimental details see the text. The fractions, blotted on to nitrocellulose membranes, were analysed with PAS (—) and Alcian Blue at pH 2.5 (---); ···, nominal percentage of buffer B.

alkylammonium chloride on a hypophase of either 6 M-guanidinium chloride or 50 mM-magnesium acetate, picked up on to carbon-coated grids, washed in ethanol (95%), shadowed with platinum/tungsten and observed with a JEOL 1200 electron microscope at 120 kV. Molecules spread from a hypophase of 50 mM-magnesium acetate were transferred into this solvent either by rapid dilution or by slow dialysis. Contour lengths of subunits and T-domains were measured with a Planix digital planimeter (Hall and Watts, Links Trading Estate, Yeovil, Somerset, U.K.). Measurements were performed on 150–300 molecules and the number-average (l_n) and weight-average (l_w) lengths calculated.

RESULTS

The samples were readily solubilized with slow stirring in 6 M-guanidinium chloride as described for mucins from human cervical mucus, chronic-bronchitic sputum and pig stomach (Carlstedt *et al.*, 1983a; Carlstedt & Sheehan, 1984). After density-gradient centrifugation in CsCl/4 M-guanidinium chloride (Fig. 1a), the mucins banded at a density of 1.41 g/ml and were quite well resolved from a small u.v.-absorbing peak occurring at a density of 1.47 g/ml, as expected for DNA. The mucin peak was pooled and subjected to density-gradient centrifugation in CsCl/0.2 M-guanidinium chloride (Fig. 1b). The mucins banded at 1.47 g/ml and were separated from residual DNA. The macromolecules thus showed

Fig. 4. Electron microscopy of tracheobronchial 'whole' mucins, subunits and T-domains

The macromolecules were spread in benzyldimethylalkylammonium chloride monolayers, picked up on carbon-coated grids and made visible by rotary shadowing with platinum/tungsten. (a) 'Whole' mucins rapidly diluted from 6 M-guanidinium chloride into 50 mM-magnesium acetate appeared either as coiled aggregates or as polydisperse, extended, meandering, linear threads. (b) When spread directly on to 6 M-guanidinium chloride, the molecules were generally untangled, with a linear but highly convoluted appearance, and were very polydisperse in length. (c) Subunits and (d) T-domains spread on a hypophase of 50 mM-

Fig. 5. Length distributions of subunits and T-domains

Subunits and T-domains were studied with electron microscopy as outlined in the legend to Fig. 4. (a) The length distributions of the subunits give l_n 410 nm and l_w 490 nm, and suggest that the molecules are heterogeneous in size. (b) T-domains are relatively homogeneous ($l_n = 140$ nm; $l_w = 160$ nm), with evidence for a subpopulation at about 100 nm. ▨, Number distributions; ■, weight distributions.

the increase in buoyant density previously observed for cervical mucins (Carlstedt *et al.*, 1983a). Data for the three pools of samples studied were very similar.

The 'whole' mucins were eluted almost exclusively in the void volume of a Sepharose CL-2B column (Fig. 2), indicating that the molecules are large, with a M_r value in excess of $(3-4) \times 10^6$. The behaviour of the major

fragments obtained after reduction (subunits) and after subsequent trypsin digestion (T-domains) on this gel is very similar to that previously observed for cervical mucins (Carlstedt *et al.*, 1983b), except that two partially resolved peaks were observed for the tracheal T-domains. It should be pointed out, however, that different methods of analysis were used in the two cases. The cervical T-

magnesium acetate appeared as randomly distributed polydisperse threads and extended rod-like structures respectively. The latter were usually found aligned over large areas of the grids, indicating poor adherence to the carbon film and/or deposition under flow or surface-tension forces.

Fig. 6. Size distribution of 'whole' mucins

Guanidinium chloride gradients were formed and run as described in the text. (a) The concentration of guanidinium chloride (○) was determined by refractive-index measurements, and the concentration of mucin (●) by PAS staining after slot-blotting, a solution of chronic-bronchitic mucins with known concentration being used as a standard. (b) The values of D_i (△) were determined by quasi-elastic light-scattering at 25 °C and a scattering angle (θ) of 20° and corrected to 20 °C and 4 M-guanidinium chloride. The corresponding values for R_g (▲) were obtained with the Stokes-Einstein equation. (c) Values of M_r (■) were determined by using data previously obtained for cervical mucins (Sheehan & Carlstedt, 1987).

domains were assayed for sialic acid, whereas a more general method (PAS) was used here. Further evidence for heterogeneity within the T-domain population was obtained by using ion-exchange h.p.l.c. on Mono Q (Fig. 3). At least three partially resolved peaks were present, and the increasing ratio of Alcian Blue to PAS response across the distribution is in keeping with the more polyanionic character of the species appearing late in the chromatogram.

The appearance of the 'whole' mucins when studied with electron microscopy differed, depending on the technique used to prepare the solutions. Samples were initially handled as described by Sheehan *et al.* (1986), i.e. stock solutions in 4–6 M-guanidinium chloride were diluted directly into a large volume of 50 mM-magnesium acetate. With this procedure, the molecules were observed both as extended thread-like particles and as coiled entangled aggregates (Fig. 4a). In contrast, slow dialysis from 6 M-guanidinium chloride into 50 mM-magnesium acetate afforded less entangled structures (results not shown), but many of the molecules were very long, suggesting that staggered side-by-side and/or end-to-end aggregations may be a feature of the distribution. Mucins spread on 6 M-guanidinium chloride showed a more uniform behaviour (Fig. 4b), dominated by randomly dispersed, flexible and coiling structures with few entangled aggregates.

The subunits showed little tendency to aggregate when diluted rapidly into 50 mM-magnesium acetate (Fig. 4c), and the molecules adopted a linear, random, configuration in this solvent. Contour length measurements yielded values of l_n and l_w of 410 nm and 490 nm respectively. The distribution (Fig. 5a) suggests that the molecules are heterogeneous.

The T-domains were observed as fully extended rod-like particles (Fig. 4d), and the alignment of the molecules on the grid suggests that they were influenced by flow and/or that they adhere poorly to the carbon film. The number- and weight-length distributions (Fig. 5b) yielded values of l_n and l_w of 140 nm and 160 nm respectively.

Rate-zonal centrifugation was used to study the size distribution of the whole mucins, and the mass distribution of the molecules in the guanidinium chloride gradient is shown in Fig. 6(a). The refractive index (necessary to determine the value of D_i with quasi-elastic light-scattering) was determined on the individual fractions in order to assess the guanidinium chloride concentration and thus the viscosity of the solutions (needed to correct data to standard conditions and to determine R_g). The value of D_i for the unfractionated mucins was $0.16 \times 10^{-7} \text{ cm}^2/\text{s}$, and those obtained for the individual fractions are shown in Fig. 6(b) together with the corresponding values for R_g . The values of D_i were not extrapolated to zero concentrations, because the

concentration-dependence is very small in this range (Sheehan & Carlstedt, 1984). Cognate values of M_r (Fig. 6b) were calculated by using data obtained previously for cervical mucins (Sheehan & Carlstedt, 1987), with the assumption that the relationship between D_i and M_r is the same for the two glycoproteins. Values for M_r of 14×10^6 and 16×10^6 were obtained for the unfractionated material and by integration over the distribution (Fig. 6a) respectively. The molecules comprised an almost unimodal distribution between M_r values from about 5×10^6 up to approx. 20×10^6 .

DISCUSSION

The mucous secretions were all from healthy non-smokers with no evidence of acute or chronic respiratory disease, and were therefore regarded as originating from a 'normal' respiratory mucosa. Although there were no signs of chronic irritation of the mucosa with a concomitant hypersecretion of mucus, the sampling technique itself might have caused an acute irritation and thus triggered secretion. The effects of low concentrations of halothane or N_2O are not known in this respect. Furthermore, the mechanical effects of inserting the tracheal tube and of the suction process could well have induced secretion. No premedication was given, specifically no cholinergic antagonists.

The mucus glycoproteins isolated from the tracheal secretions were very large and polydisperse as studied with electron microscopy, rate-zonal centrifugation and quasi-elastic light-scattering. The weight-average M_r was estimated as $(14-16) \times 10^6$, within a range of approx. $(5-20) \times 10^6$. Electron microscopy showed the molecules as linear and apparently flexible chains with a large configurational freedom. The increased tendency of the mucins to form large tangled structures when rapidly transferred from guanidinium chloride into magnesium acetate suggests that a rapid 'renaturation' of unfolded regions may cause inter-molecular associations. The contour lengths in 6 M-guanidinium chloride, although not measured on enough molecules to provide a distribution, appear to be consistent with the size range obtained by rate-zonal centrifugation, whereas some of the very large species observed in magnesium acetate are likely to be aggregates. The data obtained with electron microscopy are much in keeping with those previously reported for other respiratory mucins (Jenssen *et al.*, 1980; Slayter *et al.*, 1984; Rose *et al.*, 1984; Mikkelsen *et al.*, 1985; Sheehan *et al.*, 1986; Marianne *et al.*, 1987).

Subunits obtained after reduction were distinctly smaller (I_w 300–600 nm) than the whole mucins, and could be further fragmented into high M_r glycopeptides (T-domains) corresponding to the oligosaccharide clusters present in the macromolecules, and there must thus be regions of 'naked' polypeptide within the subunits. The T-domains were heterogeneous both in size and in charge density, and it remains an open question whether this is due to the presence of different populations of 'whole' mucins, whether a single mucin may contain different populations of subunits, or whether there are regions with distinctly different glycosylation within a subunit.

Proteoglycans/glycosaminoglycans have been identified in secretions from the human respiratory tract (Bhaskar *et al.*, 1985), from bronchial mucosa in organ

culture (Bhaskar *et al.*, 1986) and from cultured airway epithelial cells (Varsano *et al.*, 1987; Paul *et al.*, 1988). Bhaskar *et al.* (1985, 1986) were unable to detect mucus glycoproteins from the unstimulated healthy respiratory tract of man or dog. In contrast, under the conditions studied here, glycoproteins with a similar macromolecular architecture to that observed for mucins from cervical mucus and chronic-bronchitic sputum were identified. The study also shows to what extent small amounts of these macromolecules can be characterized with the availability of sensitive chemical (slot-blotting and staining) and biophysical (light-scattering and electron microscopy) techniques. It is hoped that this approach may provide the basis for future comparative studies on 'normal' and 'diseased' respiratory mucins.

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