Covalently Linked Fatty Acids in Gastric Mucus Glycoprotein of Cystic Fibrosis Patients*

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Undegraded mucus glycoprotein has been isolated in highly purified form from gastric secretion of cystic fibrosis patients. The purification procedure involved gel filtrations on Bio-Gel P-100 and Bio-Gel A-50 and lipid extractions with five mixtures of the organic solvents. The final preparation represented pure glycoprotein as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, cesium chloride density centrifugation, and lipid analysis.

Treatment of the pure and delipitated glycoprotein with methanolic KOH or hydroxyamine resulted in liberation of ester-bound fatty acids. Of the total released fatty acids, 95% were represented by hexadecanoate (36.5%), octadecanoate (48.7%), and octadecenolate (8.6%). The quantitative analysis established that, on the average, 12.2 nmol of fatty acids/mg of glycoprotein were released. The studies on cystic fibrotic glycoprotein susceptibility to proteolytic digestion indicated that fraction of glycoprotein which was resistant to pronase digestion contained on the average 33.1 nmol of fatty acids/mg of glycoprotein. After removal of the fatty acid residues from pronase-resistant glycoprotein, by treatment with hydroxyamine, the glycoprotein became susceptible to proteolytic digestion. Thus, in cystic fibrosis, the covalently bound fatty acids interfere with proteolytic degradation of mucus glycoprotein. Perhaps this is the major defect of cystic fibrosis glycoproteins and the cause of the obstruction of secretory glands and the accumulation of poorly soluble secretions.

The alteration in mucus-secreting glands and production of a poorly soluble secretion are very common and prominent features of cystic fibrosis disorder. The abnormal physicochemical properties of the secretions reflect undoubtedly the fundamental derangement in structure or metabolism of its components. Hence, numerous studies have investigated an abnormality in the metabolism of mucus glycoproteins (1-4), the properties of lysosomal glycosidases (5, 6), and the presence of unique metabolite factors (7-10) specific for cystic fibrosis, all of which should logically lead to the understanding of this enigmatic disorder. However, no consistent findings have been obtained to explain the biochemical defect in cystic fibrosis.

Considerably less attention has been devoted to the investigation of lipids in mucous secretions and the cystic fibrosis individuals (11, 12). Recently, we have demonstrated that in cystic fibrosis tracheobronchial secretion (13), submandibular saliva (14), and gastric secretion (15) exhibit elevated level of lipids. Furthermore, while studying gastric mucus from cystic fibrosis patients, we made an observation that the mucus glycoprotein contains covalently bound fatty acids. In this report, we present data regarding the content and composition of the ester-linked fatty acids in gastric mucus glycoprotein of cystic fibrosis patients. Also, we propose that the covalently attached fatty acids attribute to the proteolytic resistance of gastric mucus glycoprotein in this disease.

EXPERIMENTAL PROCEDURES

Materials—Mucus glycoprotein was isolated from gastric content of 4 cystic fibrosis individuals. Two patients (ages 14 and 20) were relatively healthy, whereas the materials from two other individuals (ages 12 and 20) were collected during the autopsies. Bio-Gel P-100, Bio-Gel A-50, and polyacrylamide gel electrophoresis reagents were purchased from Bio-Rad. Fatty acid methyl esters were standard were purchased from Supelco. Pronase, B grade, activity 69,900 proteolytic units/g at 40 °C was from Calbiochem. High performance thin layer plates, polyamide sheets, amino acid standards, and dabsyl chloride were purchased from Pierce Chemical Co. All the reagents were supplied by J. T. Baker Chemical Co.

Isolation of Undegraded Mucus Glycoprotein—Samples of gastric secretion obtained from cystic fibrosis patients were dialyzed against distilled water, lyophilized, and extracted twice with chloriform/methanol (2:1, v/v). The delipitated residue was dissolved in 2 M NaCl, applied in 100 mg portions onto a Bio-Gel P-100 column (170 × 2.5 cm) using 6 M urea as an eluent. The glycoprotein fraction recovered from the exclusion volume of A-50 gel (undegraded glycoprotein) was subjected to lipid extraction. The glycoprotein material recovered after this fractionation represented pure delipitated undegraded gastric mucus glycoprotein, as judged by gel filtration profile, equilibrium density gradient centrifugation in CsCl, SDS-polyacrylamide gel electrophoresis, and carbohydrate and protein analyses.

Mild Alkaline Methanolysis—10 mg of the thoroughly delipitated undegraded mucus glycoprotein was dissolved in 3 ml of 0.3 M methanolic KOH and incubated for 30 min at 37 °C. To incubate, 10 mmol of the internal standard (methyl nonadecanoate) was added, the mixture was acidified with methanolic HCI, and fatty acid methyl esters were extracted (3-5 times) with equal volume of hexane. The hexane phases were combined, evaporated to dryness, and dissolved in 100 μl of chloroform. The sample was analyzed for fatty acid methyl esters content and composition by gas chromatography (15). By use of quantitative mixture of standard fatty acids, the average response for each component with respect to methyl nonadecanoate was determined. This was used to convert the fatty acid area of the glycoprotein samples to nmol of fatty acids.

Pronase Digestion—The delipitated, undegraded mucus glycoprotein was subjected to pronase digestion (substrate to enzyme ratio, 30:1, w/w) in 0.15 M TRIS-HCl buffer, pH 7.0, containing 15 mm CaCl2, for 72 h at 37 °C (16). Every 12 h, the pH was adjusted to 7.0 with 2 M NaOH, and every 24 h, a new portion of predigested pronase was added.
was added. The obtained digest was applied to Bio-Gel P-100, and the glycoprotein digest was recovered. When the digested glycoprotein material was separated on Bio-Gel A-50 under conditions described under "Isolation of Undergraded Mucus Glycoprotein," the undergraded pronase-resistant glycoprotein was collected and its aliquots (10 mg each) were subjected to mild alkaline methanolysis and to deacylation with hydroxylamine. The hydroxylamine-treated material was once again treated with pronase.

Decylation of Mucus Glycoprotein with Hydroxylamine (17)–10 mg of undergraded native or pronase-resistant mucus glycoprotein was mixed with 3 ml of 1 M hydroxylamine, pH 7.0, 5 h at 22 °C. After this incubation, the sample was dialyzed against distilled water, concentrated to 5-mI volume, acidified with methanolic HCl, and extracted (5 times) with hexane. The hexane phases were combined, evaporated to dryness, and methanolyzed in 1.2 N methanolic HCl for 5 h at 80 °C. The fatty acid methyl esters were extracted from methanolaze with hexane and quantitated by gas chromatography as described under "Mild Alkaline Methanolysis." The acidified aqueous phase containing deacylated glycoprotein was lyophilized and then chromatographed on a Bio-Gel A-50 column. The undergraded decylated mucus glycoprotein recovered from A-50 gel was subjected once again to pronase digestion.

Analytical Methods—The analytical SDS-polyacrylamide gel electrophoresis, and ultracentrifugation in cesium chloride density gradient were performed according to procedures described by Laemmli (19) and Starkey et al. (20), respectively. The content of protein and carbohydrates in mucus glycoprotein was determined colorimetrically and by gas chromatography (13, 21, 22). Argentation thin layer chromatography of fatty acid methyl esters was performed on high performance thin layer plates containing 3% AgNO3 (23). The release of amino acids was monitored by dabsyl chloride procedure described by Chang and Creaser (24). Gas chromatography was performed with a Sigma 3B Chromatograph, equipped with a glass column (180 x 0.2 cm) packed with 3% SE-30 on Chromosorb W (80–100 mesh). For the analysis of trimethylsilyl derivatives of methyl glycosides, the temperature was programmed at 1.5 °C/min from 110 to 200 °C. The temperature program for fatty acid methyl esters was 140–240 °C at 2 °C/min.

RESULTS AND DISCUSSION

Cystic fibrosis was at one time considered to be an inborn error of glycoprotein synthesis (25), and in spite of many contradictions (3, 26) still seems to be the most reasonable explanation of this disease (4). However, the studies conducted until now have neither proved nor refuted this hypothesis. Obviously, the fundamental difference between glycoproteins of healthy individuals and of those affected with cystic fibrosis has not been discovered. It was suggested that the primary abnormality of the glycoprotein might be related to an increased content of fucose, thereby altering the fucose to glucosamine acid ratio and rendering the glycoproteins less soluble (3, 27). It is doubtful that some variations in the amounts of carbohydrates or their proportions to each other might account for the enormous differences in the appearance and properties of cystic fibrosis glycoproteins (2, 25, 29). Also, it seemed reasonable to believe that the lipids which are loosely associated with mucus glycoproteins might be responsible for the physicochemical properties of glycoproteins in this disease. Indeed, we have found that the amount of lipids present in gastric, salivary, and tracheobronchial secretion (15–15) are in cystic fibrosis severalfold higher than those of normal individuals. However, after extraction of lipids, the properties of mucus glycoproteins have not changed as much as we anticipated.2 After extraction of lipids, we have observed only slight improvement in solubility and pronase susceptibility of cystic fibrosis glycoproteins. Therefore, we concluded that the elevated amount of lipids in samples from cystic fibrosis patients is not the only reason for poor solubility of their mucus and inertness of glycoprotein to proteases.

To establish whether the native cystic fibrosis mucus glycoprotein contains covalently bound lipids, as found in protein of brain myelin or virus glycoproteins (28), the undergraded glycoprotein was isolated and purified from gastric secretion of these patients (Fig. 1, A and B). The isolated mucus glycoprotein was shown to be pure and free of noncovalently bound glycopeptides, proteins, and lipids by the following criteria: no other components could be detected in glycoprotein in SDS-polyacrylamide gel electrophoresis (Fig. 2, lanes 2 and 3); no protein was detected in the low density fractions of an equilibrium centrifugation in a density gradient of cesium chloride (Fig. 3); and no fatty acids were found in the final extract of glycoprotein with chloroform/methanol/water (65:35:8, v/v/v). The quantitative analyses revealed that the isolated glycoprotein contained 10.3% of protein and 88.1% of carbohydrates. Thus, purified cystic fibrosis glycoprotein was found to contain ester-bound fatty acids. The mild alkaline methanolysis of the native undergraded mucus glycoprotein resulted in release of 12.2 nmol of fatty acids/mg of glycoproteins comprised of hexadecanoate (36.5%), octadecanoate (48.7%), and octadecenoate (8.6%) (Table I). Based on quantitative determination of fatty acid content in the native undergraded and delipidated glycoprotein (12.2 nmol of fatty acids/mg of glycoprotein) and molecular weight of 2 × 106 reported for human gastric mucus glycoprotein (29), we estimate that 1 mol of native glycoprotein contains at least 24 mol of ester-bound fatty acids. The fraction of the native glycoprotein, which was resistant to proteolytic digestion and remained undegraded after 72 h of incubation with pronase, contained 33.1 nmol of fatty acids/mg of glycoproteins which amount to 66 mol of ester-bound fatty acids/mol of the glycoprotein. Presumably, the native undergraded material contained two pools of glycoprotein; one was acylated to lesser extent and still susceptible to pronase digestion, whereas the other glycoprotein pool was heavily acylated and that prevented pronase from its proteolytic action. It is possible that, to some extent, the glycoprotein heterogeneity with respect to acylation resulted from pooling the material from four individuals who differed in the degree of illness.

To substantiate the conclusion with regard to proteolytic resistance of fatty acid acylated glycoprotein the pronase-resistant glycoprotein fraction was isolated, treated under very mild conditions with 1 M hydroxylamine, and subjected once again to pronase digestion. Figs. 1, D and E illustrate this experiment, which showed that the removal of fatty acids from pronase resistant-glycoprotein rendered this material susceptible to proteolytic digestion. Since hydroxylamine at pH 7.0 is a very mild deacylating reagent (17, 28), the treatment resulted in liberation of about 90% of fatty acids (Table I). A small portion of the undergraded material, represented by the first peak in Fig. 1E, was found to contain fatty acids.

The deacylation with hydroxylamine led to release of fatty acids with no detectable degradation of glycoprotein. The aliquots of the dialyze of the hydroxylamine-treated glycoprotein were analyzed for carbohydrates (30, 31) and amino acids and peptides (24). Both the gas chromatography of trimethylsilyl derivatives of carbohydrates (13) and thin layer chromatography of dabsyl chloride derivatives of amino acids and peptides (24) showed no detectable degradation of glycoprotein. Also, the electrophoretic profile of the deacylated glycoprotein in SDS-polyacrylamide gel was identical with that of native material (Fig. 2, lane 2). Therefore, we concluded that treatment of the glycoprotein with 1 M hydroxylamine led only to hydrolysis of ester bound fatty acids, while the molecule remained otherwise intact.

Although the molecular weight of the deacylated material remained practically the same as that of untreated glycoproteins...
Fig. 1. Chromatographic profiles of gastric mucus glycoprotein from cystic fibrosis patients at various stages of purification and degradation. Bio-Gel A-50 column (170 x 2.5 cm) equilibrated and eluted with 6 M urea, flow rate 11.5 ml/h. A, sepa-
ration of the undegraded mucus glycoprotein (GI) from partially degraded glycoprotein and other components of gastric mucus. B, rechromatography of GI fraction after extraction of the noncovalently associated lipids. C, chromatography of GI glycoprotein subjected to 72-h digestion with pronase (○○○○) and then deacylation with hydroxylamine (● ● ● ●). D, undegraded pronase-resistant glycoprotein after deacylation with hydroxylamine (GIH). E, GIH glycoprotein subjected to 72 h of digestion with pronase. GIHPR represents acylated, pronase-resistant glycoprotein fraction; GIHPS represents the deacylated pronase susceptible glycoprotein fraction.

Fig. 2. SDS, 7% polyacrylamide gel electrophoresis of the gastric mucus glycoprotein at various stages of purification and degradation. Lane 1, molecular weight markers from the top: myosin (200,000), β-galactosidase (116,220), phosphorylase b (92,500), bovine serum albumin (66,000), ovalbumin (45,000). Lane 2, unde-
graded mucus glycoprotein after deacylation with hydroxylamine (300 μg). Lane 3, undegraded, native gastric mucus glycoprotein (100 μg). Lane 4, crude glycoprotein from gastric mucus before separation on Bio-Gel A-50 column (300 μg). The upper portion of the gel (spacer gel) shows that portion of glycoprotein material aggregated and did not enter the running gel.

In our opinion, this indicates that the deacylation of cystic fibrosis glycoprotein improved its solubility, and thus less of the material aggregated and formed the sediment.

Taken together, these results strongly suggest that in cystic fibrosis mucus glycoprotein solubility and proteolytic suscep-
tibility are deranged by ester-bound fatty acids. As compared to glycoproteins from normal individuals (Table 1), this alteration in glycoprotein composition inevitably leads to metabolic disturbances in the concerted process of mucus formation and degradation, accumulation of the thick insoluble mucus, and obstruction of the secretory glands. Perhaps deficient proteolytic activity found in cystic fibrosis serum (32) and the abnormal interaction of α2-macroglobulin (33) result also from excessive acylation of the substrates and not only from defective glycosylation (34). By the same token, it is very likely that various enzymes of glycoprotein nature, as well as specific glycopeptide factors, found in cystic fibrotic serum differ from their normal counterparts in having fatty acids attached to their molecules. This would explain the etiologic action of the apparently identical glycoprotein factors from normal and cystic fibrosis serum, where only factor derived from cystic fibrosis was capable to cause ciliary disfunction (10). Mainly, however, it will be important to determine whether substitution of fatty acids on glycoproteins in healthy individuals reflects the regulatory process of glycoprotein degradation and their susceptibility to protease action (35).

**Fig. 3.** Equilibrium centrifugation in a CsCl density gradient of the undegraded mucus glycoprotein after extraction of noncovalently associated lipids and rechromatography on Bio-Gel A-50 column (Fig. 1B, peak G1) (——), and after treatment with hydroxyamine (Fig. 1D, peak GII) (●—●). Each tube contained 5.5 mg of the above described glycoprotein/12 ml of 1.42 g/ml of CsCl solution. After centrifugation, the contents of the tube was fractionated into 12 1-ml portions, and protein and glycoprotein were determined. The applied samples were found to contain only glycoprotein (no protein was found in low density region). Therefore, for simplicity, A555 is depicted only. ——— illustrates the density of the gradient.

**Table 1**

| Glycoprotein | Fatty acids | Native Pronase resistant |
|--------------|-------------|-------------------------|
|              | Control     | Cystic fibrosis          | Cystic fibrosis |
| C16:0        | 47.0        | 36.5                     | 46.6          |
| C18:0        | 22.0        | 48.7                     | 66.9          |
| C20:0        | 0.5         | 0.1                      | 1.3           |
| C22:0        | 0.6         | 1.5                      | 0.5           |
| C24:0        | 5.9         | 1.8                      | 0.9           |
| C24:1        | 2.7         | 1.1                      | 0.4           |
| C18:1        | 14.5        | 8.6                      | 16.5          |
| C24:1        | 6.0         | 1.1                      | 2.2           |

nmol fatty acids/mg glycoprotein

29 ± 0.2  12.2 ± 1.4  33.1 ± 4.8  28.9 ± 4.4

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