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ISOLATION OF FATTY ACIDS COVALENTLY BOUND TO THE GASTRIC MUCUS GLYCOPROTEIN OF NORMAL AND CYSTIC FIBROSIS PATIENTS

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Covalently bound fatty acids were found in strictly purified and delipidated gastric mucus glycoprotein of normal and cystic fibrosis individuals. The susceptibility of this linkage to methanolic KOH and hydroxylamine treatment indicated the ester bond between fatty acids and glycoprotein. On the average, 2.9 nmol fatty acid/mg glycoprotein were found in normal samples, and 12.2 nmol/mg glycoprotein in samples derived from cystic fibrosis. In normal gastric mucus glycoprotein the covalently linked fatty acids consisted of hexadecanoate (47.0%), octadecanoate (22.0%), tetracosanoate (5.9%), octadecenoate (14.5%) and tetracosenoate (6.0%). In cystic fibrosis mucus glycoprotein the covalently bound fatty acids were comprised mainly of hexadecanoate (36.5%), octadecanoate (48.7%) and octadecenoate (8.6%). These data indicate that cystic fibrosis gastric mucus glycoprotein is highly acylated and perhaps this is the major defect of glycoproteins in this disease.

The understanding of secretory mucus glycoproteins in terms of their partial structures and mechanisms of their biosynthesis has been greatly advanced (1-5). However, while the basic steps in the assembly and degradation are known, a good deal more needs to be learned about the effect of the primary composition and structure on the basic function of gastrointestinal glycoproteins which is to protect mucosal epithelium from injuries (6). The rationalization of the biochemical mechanism of the protective role of gastric mucus glycoprotein would lead to better understanding of the etiology of gastrointestinal disorders. It has been proposed that the protective qualities of mucus glycoproteins are dependent on carbohydrate composition (6-9), but this is strongly argued (6,7,10). The functional significance of the negatively charged glycoproteins in hindrance of enzymatic degradation of mucus glycoproteins has been proposed (11-13) but also questioned (14-16). Thus, it still remains to be established what is responsible for the performance of

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mucus glycoproteins in health, and what changes occur in the structure or composition of these molecules in the disease. While studying the structure of gastric mucus glycoproteins, we have discovered that strictly purified glycoprotein samples contain covalently bound fatty acids. In this report, we present evidence that these fatty acids are ester bound. Also, a comparison of the fatty acid content and composition between normal glycoprotein and that derived from cystic fibrosis is being made.

MATERIALS AND METHODS

Materials - Samples of human gastric mucus were aspirated from four healthy individuals and four cystic fibrosis patients. The aspirates were filtered through ultra fine glass filter, washed free from acid with 0.1M phosphate buffer, pH 7.0, and dissolved in 6M urea for gel filtration.

Gel Filtration - Solutions of gastric mucus were introduced on Bio-Gel P100 column (170 x 2cm) and eluted with buffered 0.5M NaCl, pH 6.8. The glycoprotein excluded from P100 gel was collected, dialyzed, concentrated, dissolved in 6M urea, and applied to a Bio-Gel A50 column (170 x 2.5cm) using 6M urea as an eluent. The high molecular weight undegraded glycoprotein, contained in the exclusion volume of the column, was subjected to lipid extraction. The delipidated glycoprotein was solubilized with 6M urea and rechromatographed on Bio-Gel A-50 column. Again, the undegraded glycoprotein, contained in the exclusion volume of the column, was collected.

Extraction of Lipids - The isolated mucus glycoprotein was extracted twice with 50 volumes of chloroform/methanol (2:1, v/v) and once with: chloroform/methanol (1:1, 1:2, v/v) and chloroform/methanol/water (65:35:8, v/v/v). The consecutive lipid extracts were evaporated to dryness, subjected to acid methanolysis (1.2 methanolic HCl, 20h at 80°C) and analysis of fatty acids (17). The organic solvent extractions were continued until the extracts had no detectable fatty acids.

Mild Alkaline Methanolysis - A 10mg of thoroughly dry and delipidated mucus glycoprotein was suspended in 3ml of 0.3M methanolic KOH and incubated at 37°C for 30 min. The incubate was acidified with methanolic HCl and extracted (3 times) with hexane to obtain the fatty acid methyl esters. The hexane phases were combined, taken to dryness, dissolved in 100µl of chloroform and analyzed for fatty acids by gas chromatography (17,18). Gas chromatography of fatty acid methyl esters was performed using Sigma 3B Chromatograph equipped with 3% SE-30 column (180 x 0.2cm). The analysis was carried out at temperature program of 2°C/min from 140-240°C. By use of quantitative mixture of fatty acids, the average response for each component with respect to methyl nonadecanoate was determined. This was used to convert the fatty acids area of the glycoprotein samples to nmol of fatty acids. A 10nmol of internal standard (methyl nonadecanoate) was included in each sample analyzed for fatty acids content.

Deacylation with Hydroxylamine (19) - 10mg of the lyophilized, undegraded and delipidated mucus glycoprotein was suspended in 3ml of 1M hydroxylamine, pH 7.0, and mixed for 5h at room temperature. Then, the incubate was dialyzed partitioned three times with chloroform/methanol (2:1, v/v), and both phases were recovered. The organic phase was dried, subjected to acid methanolysis and analysis and quantitation of the fatty acids as described under Mild Alkaline Methanolysis.

Other methods - To ascertain the purity of isolated glycoprotein, an analytical SDS-polyacrylamide gel electrophoresis (20) and equilibrium density gradient centrifugation (21) were performed. The glycoprotein was measured by the modified periodic acid-Schiff base method (22) and protein by Bradford's (23) microassay procedure. The saturated and unsaturated fatty acids were separated by argentation thin-layer chromatography (17).

RESULTS AND DISCUSSION

The undegraded glycoproteins of gastric mucus from normal individuals and cystic fibrosis patients have been isolated free of partially degraded glycoproteins and non-covalently bound proteins as determined by gel filtration on Bio-Gel A-50 column (Fig. 1), SDS-gel electrophoresis (Fig. 2) and equilibrium density gradient centrifugation (Fig. 3). The non-covalent-

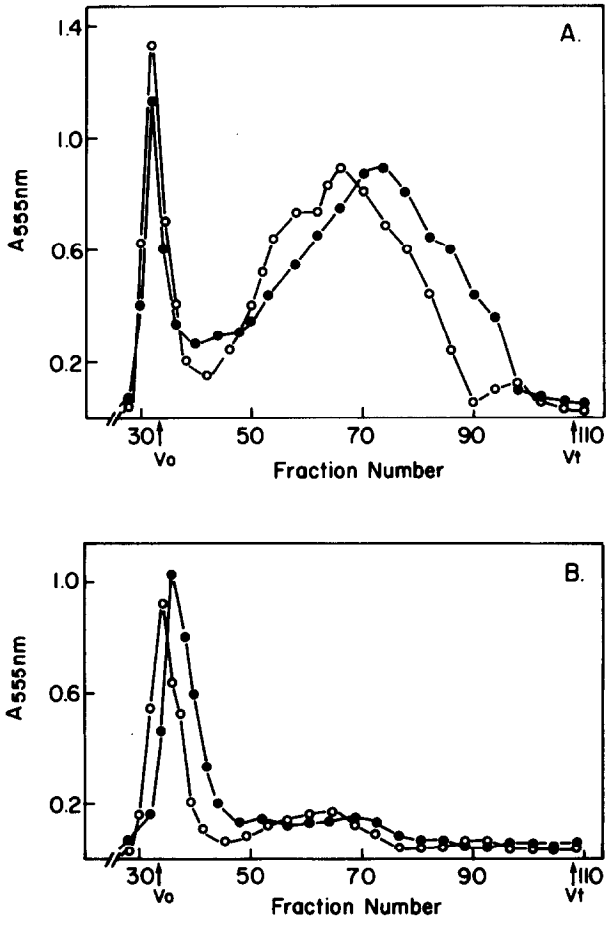

Fig. 1. Gel filtration on Bio-Gel A50 of normal (●—●) and cystic fibrosis (o—o) gastric mucus glycoprotein. Carbohydrate containing fractions from Bio-Gel P100 column were separated in 6Murea on Bio-Gel A50 column (A). The undegraded mucus glycoprotein, eluting in the exclusion volume of the column, was dialyzed, reduced in volume, subjected to lipid extraction and rechromatographed on the same A-50 gel (B). Fractions were assayed for carbohydrate (22) and protein (23) content.

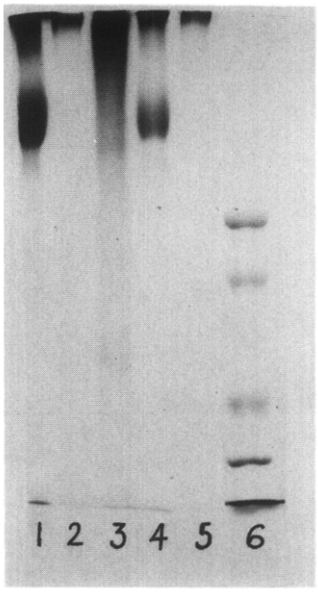

Fig. 2. SDS-polyacrylamide gel electrophoresis of gastric mucus glycoprotein from normal and cystic fibrosis individuals. Samples (300-400µg) from various stages of the purification of undegraded gastric mucus glycoprotein were electrophoresed on a 5% gel. The figure shows periodic acid-Schiff stained glycoprotein samples (lanes 1-5) and Coomassie blue G stained molecular weight standards (lane 6). Lane 1 - crude glycoprotein from normal individuals recovered after filtration on Bio-Gel P-100, lane 2 - undegraded glycoprotein of normal individuals after lipid extraction and final purification on Bio-Gel A-50, lane 3 - crude gastric mucus of cystic fibrosis patients, lane 4 - cystic fibrotic mucus glycoprotein recovered from Bio-Gel P-100 column, lane 6 - undegraded mucus glycoprotein of cystic fibrosis patients recovered after lipid extraction and rechromatography on Bio-Gel A-50 gel, lane 6 - high molecular weight protein standards (from the top; myosin (200,000), β-galactosidase (116,200), bovine serum albumin (66,200) and ovalbumin (45,000).

ly bound (associated) lipids were removed by five consecutive extractions with the increasing polarity organic solvents. First two extractions (chloroform/methanol, 2:1, v/v) removed 93-96% of loosely associated lipids, as monitored by determination of the amount of fatty acid methyl esters in the acid methanolyzed extracts. Complete removal of lipids was achieved by two additional extractions performed with chloroform/methanol (1:1 and 1:2, v/v). The fifth organic extract (chloroform/methanol/water, 65:35:8, v/v/v) contained no detectable fatty acids as judged by gas chromatography (detection sensitivity of 0.1nmol fatty acids/sample). Having ascertained the complete removal of noncovalently bound lipids from mucus glycoproteins, the samples were subjected to mild alkaline methanolysis and deacylation with 1N hydroxylamine at pH 7.0.

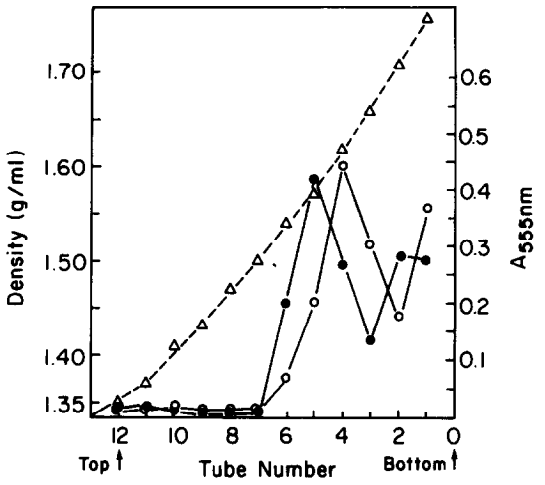

Fig. 3. Density gradient centrifugation in a CsCl gradient of the delipidated and undegraded gastric mucus glycoprotein from normal (●—●) and cystic fibrosis patients (o—o). Volumes of 1ml were collected starting from the bottom of centrifuge tubes. Each tube was assayed for content of glycoprotein (●—●, o—o) and density of the solution (Δ—Δ).

Mild alkaline methanolysis of the undegraded mucus glycoprotein of the normal and cystic fibrosis, glycoprotein resulted in liberation of fatty acids (Table I). Also, when these delipidated glycoproteins were subjected to deacylation with 1N hydroxylamine, pH 7.0, the fatty acids were released. This indicated that the fatty acid residues were ester linked to mucus glycoprotein and hence sensitive to methanolic KOH and hydroxylamine treatments, but were not removable with organic solvents alone. The identified fatty acids, in normal and cystic fibrosis glycoprotein, are shown in Table I. The patterns of these fatty acids in both glycoproteins were similar but not identical. In mucus glycoprotein of normal individuals about 23% of fatty acids were found unsaturated, whereas only 11% in cystic fibrosis. Furthermore, the cystic fibrosis derived samples contained very homogeneous population of fatty acids, of which 95% were represented by hexadecanoate, octadecanoate and octadecenoate.

Much greater differences ($p < 0.01$) between glycoprotein or normal individuals and cystic fibrosis patients were found in the amount of fatty acids released from these glycoproteins. On the average, four times more of fatty acids/mg glycoprotein were found in cystic fibrosis. Based on molecular weight of 2×10^6 reported for human gastric mucus glycoprotein (6),

Table I. Composition and content of the ester linked fatty acid of the undegraded gastric mucus glycoprotein from healthy individuals and cystic fibrosis patients.

Fatty Acid	Glycoprotein	
	Control	Cystic Fibrosis
	% composition	
C16:0	47.0 ± 2.7	36.5 ± 2.4
C18:0	22.0 ± 0.9	48.7 ± 3.0
C20:0	0.5 ± 0.0	0.1 ± 0.0
C22:0	0.6 ± 0.0	1.5 ± 0.1
C24:0	5.9 ± 0.4	1.8 ± 0.2
C16:1	2.7 ± 0.2	1.1 ± 0.1
C18:1	14.5 ± 1.0	8.6 ± 0.5
C24:1	6.0 ± 0.3	1.1 ± 0.2
nmol Fatty Acids/ mg glycoprotein[a]	2.9 ± 0.2	12.2 ± 1.4
p		< 0.01
nmol Fatty Acid/ mg glycoprotein[b]	2.6 ± 0.2	11.3 ± 1.3
p		< 0.01

Values represent the means ± SE of triplicate analyses performed on the individual samples.

[a] represent the amount of fatty acids released from glycoprotein by mild alkaline methanolysis performed with 0.3N methanolic KOH at 37°C for 30 min.

[b] represent the amount of fatty acids released from glycoprotein incubated for 5h at 22° with 1N hydroxylamine, pH 7.0.

we estimate that one mole of cystic fibrosis derived glycoprotein contains at least 24 moles of ester bound fatty acids, whereas the glycoprotein from normal individuals contains only up to 6 moles of fatty acids/mole of glycoprotein. From these results, and our studies on structure and composition of mucus glycoprotein (unpublished results), it appears that this is the major difference between normal and cystic fibrosis glycoprotein. Undoubtedly, the observed increase in number of fatty acids bound to glycoprotein of cystic fibrosis patients may significantly change the solubility of the glycoprotein and perhaps its susceptibility toward proteolytic digestion.

At this stage, however, many problems remain unresolved and in the entire vastness of research on O-glycosidic secretory glycoproteins not one communication devoted its attention to the possibility of lipid involvement in basic structure of these substances. This is the first communication on the

occurrence of the covalently bound fatty acids in the O-glycosidic secretory glycoproteins and thus its importance is in a new outlook on the primary structure of glycoproteins and their synthesis. Perhaps, this will help to unveil the basic structural defect in glycoproteins of cystic fibrosis and in the other gastrointestinal disorders. Further experiments are necessary to determine when fatty acids are attached to the synthesized glycoprotein, what is involved in the process, and how this may influence the structure and physico-chemical properties of the secretory glycoproteins.

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