In vitro Action of Gastric Mucosal Lysosomal Enzymes on Intracellular Gastric Glycoproteins

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The results show that incubation of gastric mucosal cells from rat at pH ~4.5 or in the presence of aspirin is associated with a specific increase in the activity of some acid-hydrolases. Intracellular glycoproteins, isolated by non-degradative techniques from rat or dog fundic mucosal cells, were found to be potential bio-substrates for these acid-hydrolyses. This may suggest that cleavage of the carbohydrate moieties of the intracellular and mucosal cell wall glycoproteins is a fundamental step in the development of gastric ulceration. A model for gastric lesions is proposed and discussed in the light of the results obtained.

Introduction

Gastric ulceration is one of the most widespread diseases of modern society and is attributed to many causes, in particular, to the excessive use of some anti-inflammatory drugs [1 a – c], stress and addiction to alcohol [2].

In spite of the numerous data accumulated in the literature on this subject [3], many contradictory reports [4] regarding the genesis of gastric lesions have been presented. In this respect, it has been suggested that ulceration may be the stochastic effect of several factors, namely, increase in gastric acidity [5 a – b], change in blood circulation to the gastric mucosal cells [6 a – b], decrease in gastric epithelial cell turnover [7], and possible breakdown of the mucosal barrier [8 a – d].

In case of acetylsalicylic acid induced ulceration, most of the studies showed that absorption of the drug at low pH ~2.5 resulted in gastric hemorrhage [9, 10 a – b] associated with significant changes in the activity and properties of lysosomal acid hydrolases [11]. On the other hand, when aspirin was administered at pH ~6 *i. e.*, in the ionized form, the barrier was not broken and the gastric mucosa remained almost intact [12].

These results may suggest that cellular degradation of intracellular and mucosal wall glycoproteins is probably carried out by autophagic acid hydrolases, particularly the acid hydrolases of the lysosomes. This may result in the cleavage of the bonds holding the mucosal cell structure together leading to subsequent desquamation. In this way, the submucosa and muscularis are subjected to the corrosive effect of the luminal fluid containing pepsin and hydrochloric acid [13] *i. e.*, ulceration.

Thus, laboratory degradation of such glycoproteins by lysosomal enzymes should provide a realistic model for the self-destructive processes of mucosal cells.

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Experimental

Isolation of naturally occurring glycoproteins

The viable fundic or antral gastric mucosal cells from rats or dogs were prepared as previously described [14 a – b]. These intact cells were either:

(a) incubated in Hanks or Ringer-bicarbonate solution [14 a – b], with the released glycoproteins fractionated by gel and then by ion-exchange filtration, or

(b) homogenized in urea solution (30 ml, 6 M urea/1 g wet tissue) and kept for three hours at 37 °C, with urea-extracted glycoproteins isolated by gel filtration.

Gel filtration

The glycoproteins were eluted on to a Sepharose 4 B column (100 × 3 cm) using phosphate buffer,

pH ~6, as an eluent. Glycoproteins excluded with the void volume, were collected and further purified by treatment with sodium dodecyl sulphate (1% solution), followed by repeated gel filtration on Sepharose 4 B using phosphate buffer containing SDS (1%, pH ~6). Dog fundic glycoproteins, DFG, or rat fundic glycoproteins, RFG, were further purified by ion-exchange chromatography on A-25 DEAE Sephadex to neutral and negatively charged components. Negatively charged DFG or RFG showed to contain sulphate and sialate residues. These undegraded glycoproteins were used as substrates for enzyme studies.

Protease digestion of isolated glycoproteins

DFG or RFG (0.1 g) were digested with protease as described before [15 a – d], yielding proteolysed glycoproteins, PGP.

Preparation of mitochondrial-lysosomal bound acid hydrolases

Rat or canine fundic mucosal cells were prepared as reported earlier [14 a – b]. Following subcellular fractionation [16], the mitochondrial-lysosomal fraction was resuspended in NaCl-Triton X-100, rehomogenized at 1000 rpm with ten up and down strokes and used immediately. Total activities of acid hydrolases are assembled in Table I.

Degradation of DFG and RFG by acid hydrolases

Dry DFG or RFG (0.4 g) were allowed to swell in ion-free sterile water (20 ml) at 37 °C overnight. A crystal of thymol was added to prevent bacterial growth. A kinetic run was carried out by adding mitochondrial-lysosomal homogenate to the corresponding solution of DFG or RFG and kept in a thermostated bath at 37 °C. Citrate buffer, pH ~5.6 was used in studies on the release of L-fucose, while acetate buffer, pH ~4.1 was used in following the release of D-galactose. At each time interval, one ml of the reaction mixture was withdrawn, the tube containing the aliquot was immersed in boiling water for two minutes to stop enzyme activity and to coagulate proteins, then diluted immediately with aldehyde-free ethanol (4 ml). The sample was centrifuged and filtered. The precipitate was then washed with cold ethanol (1 ml). The combined filtrate and washing was evaporated to dryness under vacuum and dissolved in water (0.2 ml) prior to the quantitative determination of the released fucose, galactose, or amino acids. Each determination was corrected for blank using homogenate incubated 37 °C. Both the neutral and charged glycoproteins obtained from A-25 DEAE Sephadex fractionation were found to be susceptible to degradation by mucosal tissue enzyme. Figs 1 a and 1 b show typical runs.

Release of D-galactose from terminal-fucose-free DFG and RFG

Removal of the terminal fucose from DFG or RFG was carried out by treatment with dilute hydrochloric acid at 4 °C. The glycoprotein solution (0.2 g/10 ml ion-free sterile water) was treated with hydrochloric acid (10 ml; 2 N) and left at 4 °C for twenty-four hours. To follow this hydrolysis at various time intervals, two ml of the reaction mixture were withdrawn, neutralized to pH ~7 and dialysed. This aliquot was then freeze-dried and suspended in water to assay for the remaining fucose, galactose, and protein. The release of terminal fucose was complete after twenty hours, some galactose, 5%, and amino acid (protein ~9%), were also released.

The fucose-free glycoprotein (0.1 g/10 ml acetate buffer, pH ~4.1) was treated with the appropriate rat or dog acid-hydrolases homogenate, and the release of carbohydrates was followed at 37 °C by the technique described above. In another experiment, proteolysed glycoprotein, PGP, was used as a biosubstrate for these acid-hydrolases, Fig. 1.

Aspirin-induced release of acid-hydrolases

Viable fundic mucosal cells from rat were incubated in Ringer medium in the presence of acetylsalicylic acid (10 mg aspirin/0.4 mg wet tissue) for four hours at 37 °C. Subcellular fractionation was carried out followed by enzyme assay of the mitochondrial-lysosomal fraction, Table I.

Release of α-L-fucose from secreted glycoproteins at different pH values

Rat mucosal cells from the fundus and antrum were prepared and incubated at pH ~7.4 and 4.5 in Ringer medium in which sodium bicarbonate was replaced by sodium citrate, for four hours. After centrifugation, the supernatant was assayed for acid glycosidases, fucosidases, fucose and protein. After dialysis, fucose and protein were assayed in order

to detect the loss of any dialysable degradation product such as fucose, fucose-bearing oligosaccharides and peptides.

Enzyme assays

The appropriate glycoside of 4-nitrophenol was used. The incubation mixture containing buffer (0.5 ml), 4-nitrophenyl glycoside (0.5 ml/5 mM) and homogenate (0.5 ml) was kept at 37 °C. Reactions arrested with trichloroacetic acid (1.5 ml; 3.3%), and kept at 37 °C for extra 5 minutes. After centrifugation, the supernatant was withdrawn to another tube and mixed with bicarbonate-carbonate buffer (1 ml) [17]. The absorption of 4-nitrophenyl anions was recorded at $\lambda_{max} = 420$, Table I.

Chemical analysis

Protein [18 a] and carbohydrates [18 b – d] were determined according to conventional methods. Sulphates were determined by IR spectroscopy.

Results

Degradation of secreted glycoproteins at high hydrogen ion concentrations

We found that incubation of fundic or antral mucosal cells in neutral medium released 1.3 units of α-fucosidase, 10.2 units of β-N-acetyl-glucosaminidase, 2 units of β-galactosidase and 280 units of Cathepsin D. Incubation at pH ~4.5 increased their release to 8.5 units of α-fucosidase, 59.3 units of β-N-acetylglucosaminidase and 300 units of Cathepsin D, but with no change in the amount of β-galactosidase. Protein loss into the medium diminished at lower pH, from 3.7 mg/100 mg tissue to 2.2 mg/100 mg tissue at pH ~4.5. These results suggest that there is a specific secretion of some acid-hydrolases during incubation, particularly in conditions of increasing acidity. Fucose was also released in fundic mucosal incubation at pH ~7.4 yielding 570 γ/100 mg tissue, and 29% was lost by dialysis. Although fucose secreted at pH ~4.5 mounted to 150 γ/100 mg tissue, yet 100% fucose was lost by dialysis.

Similarly, gastric mucosal cells from rat were incubated in Ringer medium in the presence of acetylsalicylic acid and subjected to subcellular fractionation, showed that ~50% of activity was lost from the lysosomes compared with experiments carried out in the absence of the drug, Table I. Such phenomena supports Menguy's report that glycoproteins obtained from dogs' ulcerated stomachs pretreated with aspirin, indomethacin and phenylbutazone showed a carbohydrate/protein ratio less than that of healthy dogs [8 d] .

These observations may be indicative of a mechanism in which the drug would stimulate the release of lysosomal enzymes from their sacs to degrade intracellular glycoproteins at high hydrogen ion concentrations as a step in the chain reaction leading to gastric hemorrhage. In order to study the *in vitro* effect of tissue lysosomal enzymes on gastric intracellular glycoproteins it was imperative to use DFG and RFG which are naturally occurring and

Table I. Total and bound lysosomal enzymes activities in dog and rat fundic mucosa.

Enzyme	Buffer	pH	Enzyme Activity [a]				
			DFM (Total)	DFM (ML)	RFM (Total)	RFM (ML)	Aspirin treated [c] RFM (ML)
Cath D [b]	formate	3.8	265 ± 53	70 ± 10	241 ± 50	62.3 ± 8	22.8 ± 5
AP	citrate	5	59 ± 14	23 ± 4	60 ± 15	24 ± 6	13.3 ± 2
α-Gal	acetate	4.1	1 ± 0.02	0.42 ± 0.01	0.7 ± 0.01	0.3 ± 0.001	0.1 ± 0.001
β-Gal	acetate	4.1	4 ± 0.4	2 ± 0.1	3.5 ± 0.15	1.7 ± 0.05	0.8 ± 0.07
β-NacGlu	acetate	4.3	32 ± 3	11.5 ± 2	26 ± 3	9 ± 0.08	7 ± 0.02
β-NacGal	acetate	4.8	16 ± 1	6.2 ± 0.5	12 ± 0.8	5.4 ± 0.3	2.4 ± 0.3
α-Fuc	citrate	5.6	43 ± 4	4.3 ± 0.2	40 ± 3	6.1 ± 0.9	4.3 ± 0.1

[a] Reproducibility in activity values is ±5 – 25%. Activity is expressed in units mg^{-1}, *i. e.*, nanomoles of liberated 4-nitrophenol anions min^{-1} mg^{-1} (protein). At least six tissues from different animals were assayed for enzyme activity.

[b] Assayed using denaturated haemoglobin as substrate.

[c] 10 mg aspirin/0.4 gm wet tissue.

DFM, dog fundic mucosa; RFM, rat fundic mucosa; ML, mitochondrial-lysosomal fraction; Cath D, cathepsin D; AP, acid phosphatase; α-Gal, α-Galactosidase; β-Gal, β-Galactosidase; β-NacGlu, β-N-acetylglucosaminidase; β-NacGal, β-N-acetylgalactosaminidase; and α-Fuc, α-Fucosidase.

Table II. Composition of carbohydrates of canine antral and fundic glycoproteins and glycopeptides.

Carbohydrate [a]	Antrum				Fundus			
	Major peak	PGP	DEAE Sephadex		Major peak	PGP	DEAE Sephadex	
			N	C			N	C
Fucose	5.78 11.3 *	4.92	7.92	5.28	5.96 7.53 *	5.07	8.45	4.56
Galactose	15 22.02 *	15.2	10	17	15.8 20.02 *	15.4	11.42	18.76
Galactosamine	16.5 23 *	15.97	16.2	18.4	16 19.64 *	16.2	13	20.64
Sialic acid	0.0 1.3 *	0.0	0.0	0.0	1.2 3.5 *	0.0	0.0	2.56
Sulphates	2.5 4.4 *	1.36	0.0	2.9	2.78 4.8 *	1.1	0.0	3.1

[a] Carbohydrates and sulphates units are in mg/100 mg dry mucin $\pm 1-2\%$.
* Glycoproteins extracted from dog fundic or antral homogenate by 6M urea solution.
N = Neutral glycoproteins; C = Charged glycoproteins.

isolated from gastric mucosal scraping without degradation during the preparation [19] (*cf.* glycopeptides obtained by applying potentially severe degradative techniques [15 a – d]).

Carbohydrate composition of the isolated DFG and RFG

Intracellular glycoproteins secreted from either rat or canine mucosal cells in Hanks or Ringer-bicarbonate medium, showed a low fucose/hexose molar ratio compared with those isolated by urea extraction, Table II. The former showed a similar molar ratio for both fundic and antral glycoproteins. On the other hand, the latter showed an antral molar ratio 1.5 times bigger than those of the fundus, Table II.

Degradation of DFG and RFG by intracellular enzymes

Both neutral and acidic glycoproteins, and also the original glycoprotein mixture, excluded by gel filtration, were submitted to the action of tissue glycosidases, for which they proved to be, in each case, suitable biosubstrates. Subcellular fractionation of gastric mucosa provided a suspension of lysosomes. These enzyme organelles, containing unusually high concentrations of α-L-fucosidase, were added to buffered solutions of isolated glycoproteins, both undegraded and partially degraded, to compare the roles of the latent enzymes in bracking down the cellular contents. Purified glycoproteins from rat showed different reaction kinetics from those of the dog. Both DFG and RFG lost 80% of the fucose, but the rate of loss from DFG was twice as fast, Figs 1 a and 1 b. However, RFG lost 40% of its total galactose, in a stepwise fashion, only after fucose had ceased to be released. DFG released galactose almost at the same rate as fucose, confirming that the major non-fucosyl terminal sugar was galactose. The action of dilute mineral acid, which releases labile fucosyl linkages, increased the rate of release of galactose in RFG. Degradation with a proteolytic enzyme such as pronase, also increased rates of release of galactose from RFG.

Discussion

Inspite of the mild conditions applied to isolate the intracellular glycoproteins from either rat or canine mucosal cells via incubation in Hanks or Ringer-bicarbonate medium, the isolated material showed low fucose/hexose molar ratio compared with those isolated by urea extraction. This could be accounted for by assuming that lysosomal enzymes released during the incubation have reduced the fucose/hexose molar ratio by removing 50% and 30% of the terminal fucose and hexose respectively. Thus, the prosthetic groups of the glycoproteins released in Hanks or Ringer-bicarbonate medium does not relate to the genetically controlled biosynthetized intracellular glycoprotein, *i. e.*, the released DFG or RFG in these incubation media may be degradation products of the biosynthetized one.

Thus, one can safely conclude that glycoproteins extracted by urea directly from the fresh mucosal cells are more suitable for chemical and enzymatic studies.

We found that both urea-extract DFG and RFG are degraded by acid-hydrolases, in particular, the particle bound or lysosomal enzymes obtained from the corresponding mucosal tissue. Fucose, galactose and ethanol-soluble amino acids/peptides from the glycoprotein backbone were released during twenty-four hours incubation, Figs 1 a and 1 b. The release of α-L-fucose and D-galactose was more pronounced than the release of amino acids. Interestingly, DFG and RFG showed different kinetics of release of the prosthetic carbohydrates under the effect of lysosomal enzymes. These differences are probably due to differences in the spatial arrangement of the terminal and subterminal methylpentose and hexose monomers of the prosthetic groups of the DFG and RFG [19]. Assuming that fucose is predominantly the terminal sugar in RFG, then it is logical that galactosidases would be ineffective until appreciable amounts of glycoproteins with D-galactose as the new terminal sugar would accumulate in the reaction media. Apparently defucosylation of carbohydrate chains under the effect of fucosidases would be slow at pH ~4.1. This may account for the late release of galactose under the effect of lysosomal enzymes suspension at pH ~4.1, compared with that of fucose, Fig. 1 b. This argument was further confirmed by the observation that the release of galactose from RFG pretreated with hydrochloric acid, *i. e.*, more than 90% terminal fucose removed, is faster than that from the parent RFG. Similar results were reported on the degradation of polysaccharides, mucopolysaccharides and glycoproteins by lysosomal enzymes isolated from rat kidney [20].

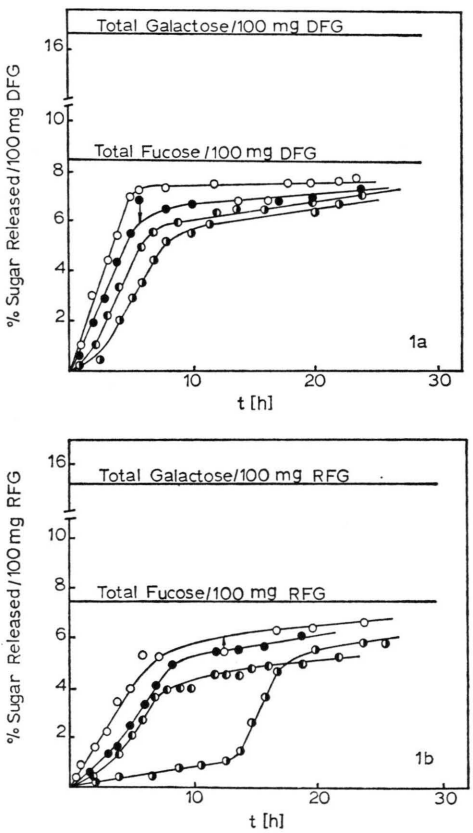

Fig. 1. Carbohydrates monomers released from:

a. DFG when treated with lysosomal enzymes from dog fundic ML homogenate.

b. RFG when treated with lysosomal enzymes from rat fundic ML homogenate. (ML, mitochondrial-lysosomal fraction.)

Release of fucose —○—○—, galactose —◑—◑— from DFG or RFG, galactose from PGP —◐—◐—, and galactose from fucose-free glycoprotein —●—●—. Experimental uncertainty is ±1—2% in each determination.

On the other hand, the release of D-galactose from DFG, Fig. 1 a, was almost parallel to the release of fucose. This may suggest that DFG has D-galactose and L-fucose as terminal sugars which could account for the earlier release of galactose. Release of galactose from fucose-free DFG was found to be slightly faster than DFG itself when treated with lysosomal enzymes. This also may be due to the increased proportion of glycoproteins, with D-galactose as the terminal sugar.

In both cases DFG and RFG pretreated with peronase, PGP, showed a faster rate of fucose liberation. This is probably due to the minimization of steric factors via removing the carbohydrate-free polypeptide chain from the glycoprotein by protease digestion, *i. e.*, a closer steric access of tissue lysosomal galactosidases to the galactosyl linkage in question.

Bearing in mind that degradation of these glycoproteins were carried out *in vitro*, the data suggest that the secretory granules and the structural glycoproteins of the gastric mucosal cells, therefore seem to provide a suitable target for autolytic processes. Accordingly, we are proposing a three-step mechanism for aspirin-induced gastric hemorrhage:

1. Aspirin inhibits oxidative phosphorylation, *i. e.*, it decreases the total energy necessary for the maintenance of the gastric mucosal cells.

2. As a result of step 1, cell and lysosomal membrane may swell and consequantly disintegrate. This leads to the release of potent digestive enzymes including fucosidase, acid-galactosidases, etc.

3. The degradation of cell structural material, secretory glycoproteins and glycoproteins of the cell membrane results from the release of lysosomal enzymes.

The proposed three-step mechanism gains some support from the observation that a deficit in the energy metabolism of gastric mucosal cells usually preceds gastric ulceration [22, 23]. Menguy *et al.* correlated the decrease in gastric mucosal energy metabolism due to gastric ischemia, to stress ulceration [22]. Similarly, Mozsik and Vizi observed a significant decrease in stomach-wall ATP in pylorus-ligated rats with concurrent development of ulcers [23]. This mechanism gained further support from experimentation on methods of inhibition of gastric lesions. Our preliminary investigations showed that reagents known to stabilise lysosomal sacs, such as lidocaine-HCl, are successful in inhibiting gastric hemorrhage in the presence of the ulcerogen, *e. g.*, alcohol, in rats.

In conclusion, our results point to:

(a) degradation of the mucigen granules and the rupture of intracellular glycosidic bonds of the mucosal cell surface membrane can be regarded as fundamental steps in the development of gastric lesions after drug induced release of lysosomal enzymes. Interestingly, asiprin showed no effect on the activity level of either α-L-fucosidase or β-D-galactosidase *in vitro*.

(b) Changes in the molar ratios of sugar monomers as well as changes in carbohydrate/protein ratio in mucosal tissue are to be expected in the early stages of gastric erosion.

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