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PROTEOLYTIC ACTIVITIES OF HUMAN CAMPYLOBACTER PYLORI AND FERRET GASTRIC CAMPYLOBACTER-LIKE ORGANISM

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The levels of proteolytic activity in cell washes, lysates and pellets of C. pylori and gastric Campylobacter-like organisms isolated from humans and ferrets, respectively, have been studied using porcine mucus glycoprotein and bovine haemoglobin substrates. The total haemoglobin degrading activity, expressed by 10^{12}-10^{13} cfu of either organism, was no greater than 3µg chymotrypsin equivalents. The mucolytic specific activity (rate of mucus peptide bond hydrolysis by bacterial protein) of the fractions tested from both organisms did not exceed 2nmol/min/mg protein. This value is 1000-fold lower than expected from published data. Electrophoretic profiles suggested that the mucolytic activity assessed by fluorimetry was insufficient to alter the quaternary structure of mucus and hence may not significantly contribute to the undermining of gastric mucus integrity.

The cause of peptic ulcer disease is unknown, although the sequence of events involved in its pathogenesis is thought to result in an imbalance between aggressive and protective factors (1). As the natural history of ulcer disease can no longer be completely explained by the "acid" hypothesis interest has been aroused by the possible role of Campylobacter pylori. There is now considerable evidence associating C. pylori with gastritis (Type B), peptic ulceration and its relapse (2-6). The nature of the impairment produced by this pathogen in gastric mucosal defence is not clear, however, Slomiany and coworkers (7,8) have identified a proteolytic enzyme elaborated by C. pylori that is capable of degrading gastric mucus. This enzymological weakening of the mucus barrier may be a mechanism contributing to the pathogenesis of peptic ulcer disease.

As part of a programme to examine the aetiological role of C. pylori in peptic ulcer disease we intended to purify and characterise the protease elaborated by C. pylori and gastric Campylobacter-like organisms

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(GCLO's) isolated from humans and ferrets respectively. Following a protocol similar to that outlined by Slomiany et al. (7) and Sarosiek et al. (8) we were unable to detect the presence of significant levels of protease secreted from cultures of either the human or ferret organisms.

MATERIALS AND METHODS

C. PYLORI PREPARATIONS

The study was conducted using cell washes, lysates and pellets of human C. pylori and ferret GCLO's derived from primary culture.

Antral biopsies, from 3 patients undergoing endoscopy for gastrointestinal investigations, were homogenised and cultured on chocolate Columbia agar (Oxoid Ltd) containing vancomycin, polymyxin B, and amphotericin for up to 7 days at 37°C under microaerophilic conditions ('GasPak' system, BBL). Ferret GCLO's were isolated from the gastric mucosa of three adult animals obtained at necropsy. Stomachs from the animals were excised and dissected to enable the mucosal surface to be gently applied to a series of agar plates. Culture conditions were as above. Presumptive identification of GCLO's was made on colonial and microscopic cellular morphologies, Gram reaction, positive oxidase and catalase reactions, strongly positive urease reaction (usually < 1 min with Christensen urea agar, Oxoid Ltd) and the inability to grow in air or under anaerobic conditions.

Organisms from primary isolation plates were suspended in prewarmed isotonic saline and the latter used to surface inoculate fresh culture plates. After incubating for 3-4 days the cells (10^{12} - 10^{13} cfu) were harvested by gently washing off the growth in saline (1ml per plate). After centrifuging the suspension (10 000 x g, 15 min) the supernatants were stored on ice and the cells resuspended in saline (2-3ml) before sonicating (MSE Soniprep, 5 x 30s with 1 min intervals on ice) and centrifuging (15 000 x g, 10 min). The lysed-cell supernatant was stored on ice. The lysed-cell pellet was resuspended in a minimal volume of saline and stored on ice. The washes, lysates and pellets were dialysed against distilled water (4°C) and lyophilised. These powders were resuspended in 50mM sodium phosphate buffer, pH 7.0 and tested for proteolytic activity.

ASSAYS

Degradation of Haemoglobin

Reconstituted samples (10µl) were incubated in a final volume of 100µl containing 50mM sodium phosphate buffer, pH 7.0 and tritiated bovine acetyl-haemoglobin (0.52mg; sp act = 8.2KBq/mg). After 10 min at 37°C, 3% (w/v) trichloroacetic acid (500µl) was added. Samples were centrifuged (11 000 x g, 5 min) and duplicate aliquots of supernatant (200µl) withdrawn for counting (2.5ml scintillation fluid). The results are expressed in equivalents (µg) of bovine pancreatic α-chymotrypsin (Type II, Sigma) which was routinely incorporated as an internal standard for the assay.

Degradation of Porcine Mucus Glycoprotein

Reconstituted samples (10µl) were incubated in a final volume of 110µl with 50mM sodium phosphate buffer/100mM NaCl, pH 7.0 containing either N-methylated (9) or non-methylated purified mucus glycoprotein (0.1mg) (10,11). After 2h at 37°C the samples were placed on ice and 200mM sodium borate buffer/200mM KCl, pH 8.5 (110µl) added. After mixing, 110µl fluorescamine in acetonitrile, 0.03% (w/v), was added followed by dilution with water (2ml). The formation of α-amino groups was measured in a Perkin-Elmer LS-5B luminescence spectrometer after 0.5h

using wavelengths of 390nm (excitation) and 475nm (emission). The results are expressed in terms of nmol α-amino groups produced per min, derived from a glycine standard curve.

Degradation of Albumin

The release of α-amino groups from N-methylated (9) or non-methylated bovine serum albumin (50μg) was determined fluorimetrically as above by substituting albumin for mucus glycoprotein.

Protein Determination

Protein was measured according to Bradford (12).

GEL FILTRATION STUDIES

These were undertaken on an Ultrogel AcA44 column (2.2 x 90cm) equilibrated in 50mM sodium phosphate buffer/100mM NaCl, pH 7.0.

Cell Saline Wash

Reconstituted saline wash (2ml) from one human isolate (2.4 x 10^{13} cfu) was chromatographed and the included protein then separated into 3 pools (fractions 1-3). Each fraction was concentrated (800-fold) by ultrafiltration (Amicon PM10) washed with distilled water, lyophilised and reconstituted before assaying proteolytic activity. Fractions 1-3 (10μl) were incubated with non-methylated samples of BSA (1mg) or mucus glycoprotein (0.1mg) in a final volume of 110μl 100mM sodium phosphate buffer, pH 7.0 for 16h at 37°C. Samples of incubate were tested for proteolytic activity both fluorimetrically and electrophoretically using the discontinuous denaturing SDS-polyacrylamide gel system of Laemmli (13). Briefly, BSA samples were reduced (dithiothreitol), electrophoresed on 7.5% slab gels and stained with Coomassie Blue, whereas non-reduced mucus glycoprotein samples were electrophoresed on 5% slab gels and visualised with periodic-acid Schiff stain (14).

Cell Lysate

The lysed-cell supernatant from the same clinical isolate was resuspended (2ml) and chromatographed as above. The included proteins were separated into two pools (fractions 1 and 2); fractions 1 and 2 comprised proteins eluting before and after BSA, respectively. Both fractions were concentrated (1000-fold) by ultrafiltration, washed with distilled water, lyophilised and reconstituted before assaying proteolytic activity.

RESULTS

HUMAN C. PYLORI

Cell Fractionation

Extensively concentrated (150-fold, 40μg protein) saline washes of C. pylori cultures did not hydrolyse haemoglobin substrate (Table 1). Concentrates (30-fold) of the lysed-cell supernatants all degraded haemoglobin; on average about 2mg lysate protein produced degradation equivalent to 2.5μg chymotrypsin. Similarly, the resuspended cell pellets degraded haemoglobin at a rate equivalent to 0.5μg chymotrypsin.

Regarding mucolysis there was no fluorimetric evidence for proteolytic degradation in the majority of fractions prepared from each isolate, however, a concentrated saline wash from one isolate and a

Table 1 Protease activity in fractions of *C.pylori* isolated from human and ferret gastric mucosa

FRACTION	HUMAN		FERRET	
	H'GLOBIN Chymotrypsin equivs (μg)	MUCUS (nmol/min/mg)	H'GLOBIN Chymotrypsin equivs (μg)	MUCUS (nmol/min/mg)
CELL WASH	0 (3/3)	1.8 (1/3)	0.15 (2/3)	1.88 (2/3)
CELL LYSATE SUPERNATANT	2.5 (3/3)	0.8 (1/3)	1.1 (3/3)	1.26 (2/3)
CELL PELLET	0.5 (3/3)	ND	0.3 (3/3)	ND

ND, not determined.
The ratios in parentheses refer to the number of samples expressing the protease activity values shown.

lysed-cell supernatant from another isolate produced new α-amino groups at the barely detectable rates of 1.8 and 0.8 nmol/min/mg protein respectively.

Gel Filtration

The 3 fractions, obtained from gel filtration of the concentrated saline wash from one isolate, were inactive in the haemoglobin assay as was the unfractionated precolumn sample, however, when assessed fluorimetrically a very small, but detectable, production of new α-amino groups from mucus substrate was observed; 1.0, 3.6 and 2.1 nmol/min for fractions 1-3 respectively. When assessed electrophoretically there was no evidence for the degradation of BSA or mucus glycoprotein by these 3 fractions. When the lysed-cell supernatant from the same clinical isolate was chromatographed, haemoglobin-degrading activities equivalent to 0.50μg and 0.28μg chymotrypsin were observed in fractions 1 and 2, respectively.

FERRET GCLO'S

Cell Fractionation

Two of three concentrated saline washes degraded haemoglobin substrate; on average the total activity was equivalent to 0.15μg chymotrypsin (Table 1). All three concentrated lysed-cell supernatants degraded haemoglobin; on average the total activity was equivalent to 1.1μg chymotrypsin. The cell pellets degraded haemoglobin on average at a rate equivalent to 0.3μg chymotrypsin. Regarding mucolysis only two of three saline washes were active, on average producing α-amino groups at a rate of 1.88 nmol/min/mg protein and only two of three cell-lysate supernatants were mucolytic at a rate of 1.26 nmol/min/mg protein.

DISCUSSION

The integrity of gastric mucus is important in maintaining the dynamic balance between aggressive and defensive factors in the stomach, so impairment of the protective function of mucus by excessive degradation may be instrumental in the aetiology of peptic ulcer disease. The correlation of C. pylori with gastritis (Type B), peptic ulcer disease and its relapse (2-6) has stimulated investigators to study the degradation of mucus glycoprotein by this pathogen. Slomiany and coworkers (7,8) have recently identified a secreted protease activity in saline washes of cultured clinical isolates of C. pylori. The protease activity described by these authors exhibited "strong" proteolytic activity not only towards a typical protein substrate such as albumin but also toward gastric mucus glycoprotein. Following a similar protocol and using the sensitive general protease substrate, tritiated-acetyl-haemoglobin, we could not identify any proteolytic activity at pH 7.0, even in cell washes concentrated 150-fold (assay protein concentrations comparable to those used by Slomiany and coworkers). Attenuation of the proteolytic activity in our samples by inhibitory components being cosecreted by the bacterium would explain the absence of haemoglobin digestion. To study this possibility a cell wash from one clinical isolate was chromatographed on Ultrogel AcA44 in an attempt to separate inhibitor from protease. The resultant 3 fractions were tested not only with tritiated-acetyl-haemoglobin, but also with purified porcine mucus glycoprotein and BSA. No activity was detected in any fraction with haemoglobin substrate, however, a very small, but measurable production of new α-amino groups was detected fluorimetrically with mucus substrate. The limited mucolytic activity evident in both the chromatographed, and other, samples is probably not significantly contributing to the undermining of gastric mucus integrity because on prolonged incubation (16h at 37°C) with mucus glycoprotein or BSA, followed by analysis on SDS-polyacrylamide gel electrophoresis, there was no visible evidence for the degradation of either BSA or mucus from the tetrameric to monomeric state.

As anticipated all the lysed-cell supernatants and pellets were capable of degrading haemoglobin, especially in the former. Therefore, a lysed-cell supernatant from one clinical isolate was analysed by gel permeation chromatography on Ultrogel AcA44, to determine the approximate M_r of the proteolytic enzymes(s) involved. Haemoglobin degrading activity was evident both in fractions of higher and lower M_r than albumin (M_r = 68 000), although the higher M_r fraction was almost twice as active as the lower M_r fraction. Since the secreted C. pylori

protease identified by Slomiany and coworkers was estimated to be of M_r < 50 000 by gel filtration, the lower M_r proteolytic activity identified in our lysates, if selectively secreted, could correspond to this enzyme. We found little evidence for the secretion of either form of proteolytic activity.

Compared to well characterised digestive enzymes eg chymotrypsin and pepsin, relatively large quantities of C. pylori enzyme protein were required for detection of proteolysis. When one considers that less than 5ng human pepsin 3 degrades haemoglobin (pH 3.0) more effectively than the total protease activity from 2.4 x 10^{13} cfu of C. pylori and that the ulcer-related pepsin, pepsin 1, is capable of significant mucolysis (35μmol/min/mg pepsin) the pathophysiological importance of the protease(s) secreted by the organism must be questioned. However, using mucus glycoprotein substrate concentrations and assay conditions similar to ours, Slomiany and coworkers (7,8) detected mucolytic activity capable of cleaving bonds at a rate of 15-75μmol/min/mg filtrate protein in contrast to our organisms which expressed 1000-fold lower activity. It is difficult to pinpoint the reason for our conflicting results; perhaps the secretion of proteolytic activity by the bacterium is sensitive to subtle differences in culture conditions eg the number of subcultures undertaken.

Animal models are needed to help establish the epidemiology and pathogenic significance of C. pylori colonisation of gastric mucosae. The high incidence of gastritis and ulceration in adult ferrets and the presence of a GCLO (15) have recently raised interest in this animal as a model. We have determined the levels of mucinase activity in GCLO, isolated from ferrets with histologically confirmed gastritis, to investigate any possible correlation between bacterial mucinase activity and gastric disease. Our results showed very low, probably insignificant levels of mucinase activity, which is very analagous to the result obtained with the human C. pylori. Therefore other mechanisms, capable of impairing the integrity of the mucus barrier, may be more important than C. pylori-dependent mucolysis in the pathogenesis of peptic ulcer disease e.g. C. pylori-mediated reduction in mucus synthesis by surface mucosal cells.

Reports on the purification and characterisation of the protease secreted by C. pylori are eagerly awaited. Furthermore, it will be interesting to see if an immune response directed toward the protease can be detected, by analogy to the IgG response found in human serum to C. pylori urease (16). Such information should undoubtedly assist in clarifying the role, if any, played by C. pylori proteases in gastritis and peptic ulcer disease.

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