Gastric Mucin Secretion from Cultured Rat Epithelial Cells

Satoru Tanji, a,b Masaki Okuda, a Rie Morishige, a and Toru Tanaka a

Faculty of Pharmaceutical Sciences and Life Science Research Center, b Josai University, I–I, Keyaki-dai, Sakado-shi, Saitama 350–02, Japan. Received October 14, 1996; accepted January 11, 1997

In order to establish the measurement of gastric mucin secreted from cultured mucous cells, rat gastric mucin was purified from secreted mucus with Sepharose CL-4B column chromatography. Gastric mucin was measured by dot blot analysis using an enzyme-linked lectin (soybean agglutinin) assay in a good concentration-dependent manner. Surface epithelial cells were dispersed by limited digestion of a rat everted stomach and collected by density gradient centrifugation with Percoll. These cells were inoculated onto gelled collagen dishes, then cultured in a medium supplemented with 10% fetal calf serum under a 5% CO 2 atmosphere in air. Changing the medium after a 2-d culture, the cells were cultured for another 3 d. During the culture, the numbers of cells each day were almost equal, but mucin contents in the cells increased, and then dropped at day 5 after inoculation. At that time, the edge of the cell layer peeled off and the cells adhered to each other. Using 2-d cultured cells, the effects of some secretagogues on mucin secretion were investigated. Carbachol, secretin, CCK-8 and prostaglandin E 2 (PGE 2 ) strongly stimulated mucin secretion, and gastrin I weakly did. However, histamine offered no stimulation.

Key words gastric mucin; epithelial cell; secretion; mucus; carbachol; secretin

Recently, by clarification of the pathogenesis of Helicobacter pylori (H. pylori) on the gastric mucosa, the importance of the roles of surface epithelial cells and the surface mucus layer on the defense mechanism was emphasized. Namely, mucus plays a role not only in the physical and chemical defense from gastric digestive juice, but also as the hostile position of H. pylori against the gastric mucosa.1) On the other hand, surface epithelial cells secrete not only mucus and bicarbonate from an apical side for this barrier, but also cytokines from a basolateral side to stimulate repair and immunological response.2) Thus, we plan to study gastric mucin secretion from surface epithelial cells for further understanding gastric defense mechanisms in vitro.

For the purpose of measuring secreted mucus from cultured cells, a highly sensitive and reliable method should be selected among the many quantitative methods for measuring mucus. A lectin or a monoclonal antibody to mucus is the recommended tool for our purpose. Gastric mucus is a group of glycoprotein, mucin, having a molecular weight of 2–44×10 6 Da.3) The chemical structures of human gastric mucins, MUC5 and MUC6, were partially determined in tandem repeats of the core protein and oligosaccharides.4,5) On the basis of several reports on rat mucin, we selected a lectin for its measurement.

Since Soll6) succeeded in isolating parietal cells and showed their functions, chief cells, gastrin cells, enterochromaffin-like (ECL) cells and mucous cells were isolated from the gastric mucosa.7) However, because of the leakage of mucin, the isolation of mucous cells in a fully functional state was difficult. Then, we utilized the characteristic that mucous cells adhere to collagen gel during culture.

In terms of mucus secretion, two possible causes are known: one is receptor-mediated secretion, the other is nonspecific, induced by necrotizing agents or inflammation.8) However, it is not known whether nonspecific irritants act on mucous cells directly or via a certain mediator indirectly. Our aim is to establish a method for measuring mucin secretion from cultured epithelial cells and to examine the effects of several secretagogues including a candidate of mediators of irritants on cultured epithelial cells.

MATERIALS AND METHODS

Materials Sepharose CL-4B and Percoll, and Immobilon-PVDF transfer membrane were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden) and Japan Millipore, Ltd. (Tokyo), respectively. Soybean agglutinin (SBA), sheep anti-soybean agglutinin IgG and horseradish peroxidase-avidin D (HRP-avidin D) were purchased from Vector Laboratories, Inc. (U.S.A.). Carbamylcholine chloride (carbachol) and bovine serum albumin (BSA), were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A.), and Disperse I and cholecystokinin octapeptide (CCK-8), gastrin I and secretin were from Godo Shusei Co. (Tokyo) and Peptide Institute, Inc. (Osaka), respectively. All other reagents were of the best commercial quality available.

Purification of Rat Gastric Mucin The stomachs were excised from Wistar rats under urethane anesthesia. Everted stomachs were rinsed and wiped, then incubated in phosphate buffered saline (pH 7.3) for 1 h and gassed with 95% O 2 and 5% CO 2 . Secreted mucus was collected with a cell scraper and freeze-dried after being dialysed against distilled water. Collected mucin was purified according to the method of Goso and Hotta.8) Briefly, mucin was homogenized with 6 μm guanidine hydrochloride (pH 7.2) containing 2% Triton X-100, 10 mm EDTA, 2 mm N-ethylmaleimide (NEM), 1 mm phenylmethylsulfonyl fluoride (PMSF), 0.15 mm pepstatin A and 50 mm Tris, and extracted by stirring for 15 h at 4 °C. Mucin solution was applied on a Sepharose CL-4B column and eluted with 4 μm guanidine hydrochloride containing 0.5% Triton X-100, 10 mm EDTA, 2 mm NEM, 1 mm PMSF, 2 mm benzamidine hydrochloride, 100 mm 6-amino hexanoic acid and 50 mm Tris (pH 7.2).

Measurement of Gastric Mucin A dot blot analysis was used for the measurement of mucin.8) Samples or
various concentrations of purified mucin were applied in a microfiltration apparatus (Immunodot, Atto Co., Ltd., Tokyo) and adsorbed on a sheet of polyvinylidene fluoride (PVDF) membrane by aspiration. After blocking the blotted membrane with BSA solution and washing it with buffer, mucin was detected by an enzyme-linked lectin assay (ELLA). Briefly, the membrane was incubated with SBA solution at room temperature for 30 min and washed. The same procedures for biotinylated-anti SBA IgG and HRP-avidin D were carried out for 30 min, respectively. Enzyme activity was detected with 0.03% H2O2 and 0.02% 3-aminophenyldiazirine as substrate.

**Isolation and Culture of Rat Epithelial Cells** Rat gastric epithelial cells were obtained by the method that we established. Briefly, the stomachs from anesthetized rats were resected and rinsed with ice cold phosphate buffered saline. The resected sacs of the stomachs, in which medium B was injected, were immersed in medium A containing Disase I (1000 U/ml) and gassed with 95% O2 and 5% CO2 at 37°C for 1 h. The stomachs were transferred to medium A with 0.5 mM EDTA. Mucosal cells were gently isolated by pipetting them from the surface of the gastric mucosa, followed by filtration through a 150-mesh nylon filter. Cells were collected by centrifugation at 50 × g for 3 min and resuspended in medium B. Surface epithelial cells were separated as a precipitate by density gradient centrifugation with 15% Percoll at 300 × g for 5 min. Obtained cells (2 × 106 cells/dish) were inoculated on plastic dishes (35 mm diameter) after mixing with collagen gel, then cultured in a mixture of Dulbecco’s modified Eagle’s minimum essential medium and Ham’s F-12 (1:1) supplemented with 10% fetal calf serum (FCS) and 100 μg/ml gentamicin sulfate at 37°C in a CO2 incubator.

Medium A: 0.5 mM NaH2PO4, 1 mM Na2HPO4, 20 mM NaHCO3, 70 mM NaCl, 5.0 mM KCl, 11 mM glucose, 25 mM HEPES, and 1.0% BSA (pH 7.3).

Medium B: 1.0 mM CaCl2, 1.5 mM MgCl2, and 0.1% BSA in medium A instead of 1.0% BSA.

**Assay of Mucin Secretion** At the 2nd day after inoculation of epithelial cells, the dishes were washed by medium B and filled with medium B for 15 min at 37°C in a CO2 incubator. A mucin secretion assay was carried out by adding the secretagogue to the culture dishes for 60 min in a CO2 incubator, because stable results were obtained at that time. A certain aliquot of culture medium was taken for the mucin measurement, after mixing by gently inclining the dishes. Total mucin was measured after sonication of the frozen and thawed cells.

**Statistcal Analysis** Statistical significance of the difference between basal values and the stimulated ones was determined by analysis of variance.

**RESULTS**

**Standard Curve of Gastric Mucin** Mucin collected from the surface of the resected stomachs was applied on a Sepharose CL-4B column. A broad mucin peak that was detected by ELLA, and three protein peaks were seen. The first protein peak which overlapped with mucin was collected and eluted again. Figure 1 shows an elution profile of the rechromatography of rat gastric mucin. The shadowed peak in Fig. 1, which was eluted just after the position of a void volume, molecular weight about 1 × 10^7 Da, was collected and freeze-dried after dialysis. The chemical components of the mucin were protein and sugar, as measured by a phenol/H2SO4 method, and the ratio of sugar was 73%. We used this mucin as the standard. The standard curve of the mucin is shown in Fig. 2. Over 3 × 10^-9 g/dot of mucin could be measured by this method.

**Characterization of Cultured Gastric Epithelial Cells** About half of the inoculated gastric epithelial cells adhered to the gelled collagen dishes in 2 d. Changing the culture medium every 2 d after inoculation, the cells were cultured for another 3 d. More than 90% of the cultured cells were periodic acid Schiff (PAS) reaction positive throughout the culture (Fig. 3). At day 5, the edge of the cell layer peeled off and the cells adhered to each other. These cells only slightly proliferated during culture. The mucin content in the cells increased up to day 4, then dropped at day 5 (Fig. 4). Since 2-d cultured cells showed good adhesion on the dishes and were shown to be PAS-positive cells, we used them for a mucin secretion experiment.

**Effects of Some Secretagogues on Mucin Secretion from Cultured Epithelial Cells** Effects of carbachol, histamine, gastrin I, CCK-8, secretin and prostaglandin E2 (PGE2) on mucin secretion are shown in Fig. 5. Although we carefully took the samples for a mucin measurement, values were widely scattered because of the insolubility...
and stickiness of the mucin. Histamine did not stimulate mucin secretion, but gastrin I weakly stimulated it, although the maximum response to gastrin I was lower than half that to other secretagogues. On the other hand, carbachol, CCK-8, secretin and PGE₂ strongly stimulated mucus secretion, and their ED₅₀ were $7.4 \times 10^{-8}$, $8.7 \times 10^{-11}$, $1.3 \times 10^{-10}$, and $7.1 \times 10^{-10}$ M, respectively. These chemicals stimulated mucin secretion in a dose-dependent manner, and a decrease in the response was seen in the high concentration of secretin.

**DISCUSSION**

Gastric mucin was purified according to the method described by Goso and Hotta. However, we strictly selected the source of mucus; we collected the secreted mucin from the everted stomachs by incubation in a medium. Therefore, the contaminants in the mucin fraction were thought to be few, such as pepsinogen or surface cell debris. Since mucin was insoluble, a micro-quantitative measurement of mucin is limited. Sometimes, mucin secretion has been evaluated by the release of incorporated $^3$H-glucosamine. The dot blot analysis is an easy and direct measurement of finely dispersed mucin. We could measure $3 \times 10^{-8}$ g of mucin using the dot blot analysis. Sensitivity of the dot blot analysis is sufficient to measure mucin secreted from cultured epithelial cells.
There are several papers which dealt with the culture of rat gastric epithelial cells.\textsuperscript{10--14} Terano, et al.\textsuperscript{11} reported that cultured cells that were isolated from infant rat gastric mucosa showed the characteristics of epithelial cells. Kinosita, et al.\textsuperscript{12} showed that proliferating cells, separated from the adult rat gastric mucosa by a elution method, differentiated to epithelial mucous cells. We also tried to separate the surface epithelial cells from adult rat gastric mucosa and culture them. Isolation of the cells was accomplished by a combination of restricted digestion of the surface of the gastric mucosa and density gradient centrifugation. Because good adhesion of the cells to gelled collagen dishes was seen 2 d after inoculation, the culture medium of inoculated cells was changed and unattached cells were discarded. We were able to obtain about 90% of PAS positive cells by these procedures. The mucin content and numbers of cells in dishes during the culture were almost equal up to day 4 after inoculation, but the content of mucin in the cells dropped at day 5. The cells that we used in this paper seemed to be surface epithelial cells, rather than proliferating cells.

The effects of some secretagogues on mucin secretion were tested. Mucin secretion from epithelial cells was stimulated by carbachol, gastrin I, CCK-8, secretin and PGE\textsubscript{2}. Since carbachol, gastrin I and CCK-8 are known as acid secretagogues, it is easily recognized that they stimulated mucin secretion. However, it is curious that secretin and PGE\textsubscript{2}, inhibitors of acid secretion, also stimulated mucin secretion, whereas histamine did not. Keater and Hanson reported that secretin stimulated radiolabelled mucus secretion from isolated rat mucous cells, but histamine did not.\textsuperscript{10} In addition, direct measurement of gastric surface mucus gel thickness was developed, in which the stimulating effect of PGE\textsubscript{2} was shown.\textsuperscript{15,16} It is also known that perorally administered necrotizing agents induce mucin secretion.\textsuperscript{15} As PGE\textsubscript{2} is a chemical mediator of irritants or inflammation,\textsuperscript{17} that PGE\textsubscript{2} is a mucin secretagogue is acceptable. It is still curious that histamine, another important chemical mediator of inflammation, did not stimulate mucin secretion. However, in order that parietal cells can exclusively use histamine, a potent acid stimulant, mucous cells may not have the histamine receptor. It seems likely that mucus secretion has no correlation with acid secretion.

Seidler, et al.\textsuperscript{18,19} reported that mucin secretion from the organ culture of rabbit gastric mucosa, which was evaluated by measuring released radiolabelled materials, was stimulated by A23187, 12-O-tetradecanoylophorbol-13-acetate, and forskolin. Yasaka and Tsukamoto\textsuperscript{20} directly measured mucus secretion with a microscopic method by analyzing video images, and showed that carbachol and forskolin stimulated it. Taking all of these results into consideration, it is suggested that mucus secretion induced by physiologically active substances is mediated by phosphatidilinositide turnover (intracellular Ca\textsuperscript{2+} increase and protein kinase C activation) or a cAMP-associated pathway.\textsuperscript{21} However, there is only slight evidence that mucus secretagogues act as their own receptors on mucous cells, because of methodological difficulty in showing that a receptor specific antagonist ought to inhibit agonist stimulated mucus secretion.\textsuperscript{19} At any rate, chemicals that stimulated mucin secretion were similar to those stimulating peptisogen secretion from chief cells, as was reported in our papers.\textsuperscript{22,23} In this paper, we showed that secretagogues induced dose-dependent mucin secretion from cultured rat epithelial cells using an enzyme-linked lectin assay.

REFERENCES