Mitogenic Response of Canine Fundic Epithelial Cells in Short-term Culture to Transforming Growth Factor α and Insulinlike Growth Factor I

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Abstract

We report methods allowing the culture of rapidly dividing gastric epithelial cells to investigate the regulation of mucosal cell replication. Cells from canine fundic mucosa were dispersed by enzyme treatment, enriched by filtration and elutriation, and cultured on collagen gel in DMEM/F12 medium. After 48 h, > 95% of the cells displayed immunoreactivity with antibody to cytokeratin, an epithelial marker. The cells formed confluent monolayers by 72 h with a transmembrane resistance of 1,600 ohm · cm^2 when mounted in a Ussing chamber indicating retention of epithelial cell characteristics. Calf serum (0.1–2%) produced a dose-dependent mitogenic effect evident by increases in [^{3}H]-thymidine incorporation into acid-precipitated material and in cell number. After an 18–24-h incubation with [^{3}H]-thymidine, ~ 55% of the cells cultured in 2% serum showed evidence of DNA synthesis by autoradiography and all of the replicating cells were cytokeratin positive. Using comparable culture conditions, a similar proportion of cells incubated for 18–24 h with bromodeoxyuridine displayed nuclear anti-bromodeoxyuridine immunoreactivity, thus indicating that over half of the cells in these cultures synthesized DNA during this period. As with serum, epidermal growth factor and transforming growth factor alpha (TGFα) (10 pM to 1 nM), insulin (10 nM to 1 μM) and insulinlike growth factor-I (IGF-I, 1–100 nM) increased [^{3}H]-thymidine uptake. The greater potency of IGF-I, compared to insulin, suggests the presence of IGF-I receptors. We conclude that this culture preparation is composed of fundic mucosal epithelial cells and contains a predominance of dividing epithelial cells. EGF/TGFα and IGF-I are potential factors directly regulating proliferation of fundic mucosal cells. (*J. Clin. Invest.* 1991. 87:1716–1723.) Key words: gastric mucosa • cell separation • cell growth • epidermal growth factor • peptic ulcer

Introduction

The gastric mucosa is a rapidly proliferating tissue, being renewed by replicating cells present in a proliferative zone located in the isthmus (neck) of the gastric gland. The migration time of cells from the progenitor zone to the mucosal surface (the time for total replacement of surface epithelial cells) is ~ 3 d in the rat and 4–6 d in human and dog (1). Monitoring [^{3}H]-thymidine (THM)[1] labeling in vivo, mucous neck cells were confirmed to rapidly replenish sloughed surface epithelium, whereas many fewer cells migrated downward into the gastric glands to replace parietal and chief cells. Chief cells may also undergo division at a slow rate, thus appearing to constitute a separate slow-cycling replicating cell pool (2, 3). Immature parietal cells, located in the upper part of the gastric glands, also appeared to incorporate thymidine (4), whereas mature parietal cells showed no such evidence of division. Mucous neck cells and immature parietal cells have been hypothesized to be a single cell type in different stages of proliferation, migration, and differentiation (4). Although the replenishment of the gastric and duodenal mucosa is of obvious critical importance to maintaining mucosal integrity in the normal state and in response to injury, knowledge regarding regulation of epithelial cell growth remains limited. In vivo models have been utilized to investigate factors regulating mucosal growth. For example, in the rat, EGF was found to stimulate [^{3}H]-THM incorporation into DNA (5–7) and to increase gastric mucosal weight, thickness, and parietal cell mass (2). Although these findings indicate that EGF has trophic effects on stomach in vivo, the cellular site and mechanism of action remain uncertain.

Several cell culture models have been reported for gastrointestinal mucosal cells. Harvesting cells by mechanical methods has allowed culture of normal human epithelial cells from colon, small intestine, and stomach (8). In these studies cells grew in suspension in clusters, rather than as adherent cells in monolayers. Proliferation was observed in the center of the clusters, and epithelial characteristics (e.g., junctional complexes, staining for cytokeratin) were reported. Conditioned medium induced cell growth, but responses of these cells to specific growth factors were not reported. Fetal rabbit gastric epithelial cells consisting mainly of surface mucous cells (50–60%) have been observed to proliferate in primary culture and form monolayers within a week (9). However, this model required the addition of 10–20% calf serum for cell proliferation, and the proliferating cell population was not well characterized. Nontransformed epithelial cell lines have been established from rat intestine (10) and human colon (11). Cultures of well-differentiated transformed cells have been established from human gastric carcinoma (12).

The goal of our present studies is to investigate the regulation of growth in normal gastric mucosa. The available culture models for gastric mucosa do not appear to permit detailed study of the regulation of proliferation by specific growth factors. Furthermore, since alterations in control mechanisms may occur with either transformed or nontransformed cell

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Received for publication 25 June 1990 and in revised form 31 October 1990.

The Journal of Clinical Investigation, Inc.
Volume 87, May 1991, 1716–1723

1. *Abbreviations used in this paper:* BrdU, bromo-deoxyuridine; DAB, 3.3'-diaminobenzidine; EGF, epidermal growth factor; IGF-I, insulinlike growth factor; I_{sc}, short circuit current; MRC, fundic mucosal replicating cells; PDGF, platelet-derived growth factor; R_0, serum-free, growth factor–free medium; TGFα, transforming growth factor α; THM, thymidine.

lines in continuous culture, thus limiting extrapolation to normal mucosa, we have chosen to investigate growth regulation in mucosa utilizing primary cultures of normal canine gastric mucosa. Our present methods were adapted from studies with endothelial cells from canine fundic mucosa (13) in which intact capillary fragments were enriched by selective filtration and elutriation. When we placed these preparations in culture, cells bearing the endothelial marker factor VIII–related antigen (13) became undetectable between 24 and 48 h in culture. However, a rapidly proliferating cell type was present, which displayed epithelial, rather than fibroblastic or endothelial, characteristics. We now report the methods used for this preparation and present data that receptors for EGF/TGFα and IGF-I induce a mitogenic response in gastric mucosal epithelial cells.

Methods

Dispersion, cell separation, and culture

The canine fundic mucosa was dispersed with collagenase and EDTA (14), filtered sequentially through coarse (62 threads/inch) and fine (195 threads/inch) nylon mesh. Material retained on the second filter was suspended in HBSS containing 0.1 mg/ml dithiothreitol and 0.01 mg/ml DNase. This cell suspension was elutriated primarily to eliminate bacteria (15). Cells ($2–3 \times 10^8$) were loaded into a model J 5.0 elutriator rotor (Beckman Instruments, Inc., Fullerton, CA) at a flow rate of 87.5 ml/min and an rpm of 2,000. After a 500-ml wash at a flow rate of 100 ml/min, a fraction was collected at a flow rate of 200 ml/min and an rpm of 1,460, thus including cells with apparent cell diameters ranging from 15 to 23 μm. This cell fraction was then suspended in our basic culture medium (R_0), composed of DMEM/Ham's F-12 (Gibco, Grand Island, NY) in a 1:1 mixture, supplemented with 20 mM Hepes (Calbiochem Corp., La Jolla, CA), 50 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA). R_0 refers to medium without added growth factors or serum, which were only added as indicated. Calf serum (2%, Gibco) was added to this R_0 medium for the initial overnight culture in which cells (1.5×10^5 cells in 0.5 ml) were plated onto polymerized type I rat tail collagen in 24-well tissue culture plates (Flow Laboratories, McLean, VA) for growth studies, or Lab Tek slides (Nunc Inc., Naperville, IL) for autoradiography and histochemistry. After overnight culture, the standard protocol was to remove this medium, wash the cells twice in fresh R_0 without added GF or serum. Serum, EGF (Amgen Corp., Thousand Oaks, CA), TGFα (Bachem, Inc., Torrance, CA), insulin or IGF-I (Bachem, Inc.) were added for the subsequent culture period, only as indicated. Petri dishes (35 mm, Corning Glass Works, Corning, NY) were also used for some histochemistry studies.

Transepithelial electrophysiology

Elutriated epithelial cells (4.0×10^6 cells in 2 ml per 35 mm petri dish) were plated on collagen-coated filters (type SS, Millipore Corp., Bedford, MA) and cultured in R_0 with 2% calf serum. These cultures formed confluent monolayers after 72 h. These filters were mounted in Ussing chambers and electrophysiological properties of the monolayers studied, as previously described (15, 16).

Immunohistochemistry

Immunohistochemical staining was performed with cells cultured for 24–48 h after Bouin's fixation using the avidin-biotin-peroxidase complex (ABC) method (17). Immunohistochemistry was performed using monoclonal antibodies to the epithelial cell marker human cytokeratin (AE1/AE3, 1:50, Boehringer Mannheim Biochemicals, Indianapolis, IN) (18), H^+/K^+-ATPase (19), and pepsinogen I (20, 21). The latter two antibodies were kindly provided respectively by Dr. A. Smolka (Charleston, SC) and Dr. I. M. Samloff (Sepulveda, CA), respectively. Rabbit polyclonal antibodies to factor VIII-related antigen was obtained from Dr. R. Benson (Albany, NY). Second antibodies are biotinylated and visualized with a peroxidase complex and DAB (3,3′-diaminobenzidine, Sigma Chemical Co., St. Louis, MO) dye. 200 cells in each of five fields were counted on coded slides by two observers unaware of the code.

Measures of cell replication

Cell number. Cells were detached from the culture wells by treating with trypsin-EDTA (Gibco) for 20 min. Cells were then counted in triplicate in a particle data counter.

Autoradiography of [^{3}H]-THM incorporation. Cells after overnight culture on collagen-coated Lab Tek slides were incubated with [^{3}H]-THM (2 μCi/ml, New England Nuclear, Boston, MA) for 18–24 h. Cells were washed and fixed with Bouin's solution. Slides were dipped with emulsion (type NTB2, Eastman Kodak Co., Rochester, NY) and exposed for 2–3 wk at 4°C. Slides were then developed in Kodak D-19 and counterstained with hematoxylin.

BrdU incorporation. DNA synthesis was measured by determining the nuclear uptake of the thymidine analogue BrdU (Sigma Chemical Co.). After overnight culture, cells were treated with 10 μM BrdU for an additional 18 h, washed, and fixed with ethanol. Immunofluorescence was performed using a monoclonal antibody to BrdU (Becton Dickinson & Co., Salt Lake City, UT), with a FITC-conjugated second antibody.

Cell mass. Cell mass was determined by measuring β-hexosaminidase, a lysosomal enzyme. Cells were incubated with exogenous substrate (*P*-nitrophenyl-*N*-acetyl-B-D-glucosaminide) (Sigma Chemical Co.) at 37°C for 30 min. The reaction was stopped by adding glycine/EDTA (pH 10). The resulting product in the media was spectrophotometrically determined (OD at 405 nm) (22).

[^{3}H]-THM incorporation. Epithelial cells were plated onto 24-well plates in R_0 with 2.0% calf serum for overnight culture. Cells were then washed and incubated for an additional 24 h in fresh R_0 medium in the presence of [^{3}H]-THM (0.5 μCi/ml), with EGF, TGFα, insulin, IGF-I, or serum added as indicated. Cells were washed in PBS, 10% TCA (4°C) was added and replaced for two 10-min periods. The material precipitated in TCA was solubilized in NaOH and radioactivity counted in a model 7500 liquid scintillation counter (Beckman Instruments, Inc.). For some studies, as indicated in the legends, we have inserted an 8–12-h additional incubation period in R_0 before growth factor and [^{3}H]-THM was added.

Combined autoradiography and immunohistochemistry

Cells cultured in Lab Tek slide chambers were incubated in [^{3}H]-THM, as noted above for the autoradiography studies. After fixation in Bouin's, immunohistochemistry using the ABC method was performed to demonstrate cytokeratin. Autoradiography for [^{3}H]-THM was then completed, as noted above.

Results

Cell separation, culture, and identification

Methods for preparing fundic replicating cells were developed from those used for mucosal endothelial cells (13) in which the crude enzyme-dispersed cell preparation was filtered. For culture studies the mixed population of single cells and gastric glands, and capillary fragments retained on nylon mesh was elutriated. After elutriation, this preparation was characterized by examining slides prepared by cytocentrifugation, stained with periodic acid Schiff (PAS) reagent (23) or stained by immunohistochemistry. At the time of plating, of the cells examined, 19±6% of the cells reacted with antibodies to H^+/K^+-ATPase, and 40±8% contained pepsinogen I–like immunoreactivity. On PAS staining, 23±6% contained positive granules typical of mucous cells. Capillary fragments were also found in these cytospins, and 22±7% of the cells reacted with antibodies to factor VIII–related antigen (mean±SE, n = 3) (Table I). These preparations were cultured in 2% calf serum in Lab Tek

Table I. Cellular Composition of Cultured Fundic Mucosal Epithelial Cells

Time of culture	Pepsinogen (n = 4)	H^+/K^+-ATPase (n = 4)	PAS (n = 3)	Factor VIII-related Ag (n = 3)
h	%	%	%	%
0	40±8	19±6	23±6	22±7
24	45±9	20±6	—	15±7
48	39±11	20±7	—	<1

At the time of plating (T = 0), cytospins were used for immunohistochemistry. At T = 24 or 48 h, cultured cells on Lab Tek slides were used for immunohistochemistry. Results, the mean±SE, are expressed as the percentage of the total cell population. The ABC method was used for all antibodies, except that immunofluorescent was used for factor VIII-related antigen (Ag).

slide chambers, and then the cellular composition was examined by immunohistochemistry after 24 and 48 h (Table I). At these two time points, 45±9% and 39±11% of cells displayed immunoreactivity with pepsinogen I, and 20±6% and 20±7% were reactive with the H^+/K^+-ATPase antibody. The PAS stain did not release typical positive granules in the cultured cells, so this marker was not quantitated. These methods did not prove useful for culturing endothelial cells, which was our initial goal; the proportion of cells reactive with antibody to VIII-related antigen decreased to 15±7% at 24 h and < 1% at 48 h (Table I). However, these cultures prepared by these methods did contain a rapidly replicating cell population, which we have found to be of epithelial origin.

Epithelial nature of the cells

Several findings established the epithelial nature of the rapidly dividing cells in these cultures. Phase contrast microscopy of cultures after 24, 48, and 72 h (Fig. 1) indicated that the cells adherent to the culture substrate had the characteristic cuboidal morphology of epithelial cells. Furthermore, confluent monolayers formed by ~ 72 h (Fig. 1). The polarized, epithelial nature of monolayers was demonstrated using electrophysiological methods (15, 16) (Fig. 2). Monolayers formed on filters and mounted in Ussing chambers had a "transepithelial" resistance of 1,600±450 ohm · cm^2 (mean±SE, n = 6); this high resistance could only occur with the formation of tight junctions between the cells of the monolayer. When the membrane was treated on the basolateral surface with 8-bromo-cAMP (0.1 mM), the short circuit current increased from 1.2 to 5.4 μA, indicating the vectorial transport that is characteristic of polarized epithelia.

The presence of cytokeratin fibers provides a specific marker for epithelial cells; immunohistochemistry was used to detect cytokeratin (18). After 48 h in culture, 95±5% (mean±SE, n = 4) of the cells examined stained positively using a cytokeratin antibody (Fig. 3, as shown in cytoplasmic pigment). Furthermore, by combining autoradiography to detect the cells that had incorporated [^{3}H]-THM into DNA with immunohistochemistry for cytokeratin, we found that essentially all of the cells that had synthesized DNA in culture (identified by grains over their nuclei) were cytokeratin-positive (Fig. 3). We conclude that the formation of monolayers with high resistance and polarized transport and the presence of specific markers establishes the epithelial nature of the replicating cells in these cultures. We thus refer to the replicating epithelial cells in these preparations as fundic mucosal replicating cells (MRC).

Assessment of cell replication in culture

Cell number. Replication of cells in these cultures was studied by counting the number of cells adherent to the plates using phase contrast microscopy (Fig. 1) and by removing cells from the plates using EDTA/trypsin treatment and counting with a Particle Data Counter (Fig. 4 *A*). With both of these techniques, the number of cells cultured in the presence of 2% calf serum increased over 72 h in culture, indicating the occurrence of cell replication in these cultures.

[^{3}H]-THM and bromodeoxyuridine incorporation into DNA. DNA synthesis was demonstrated by incubating cells with 2 μCi/ml [^{3}H]-THM, followed by autoradiography to identify and quantify the replicating cells present in these cultures. With an 18–24-h exposure to [^{3}H]-THM, we found that 55±9% (mean±SE; n = 5) of the cells present displayed radioactivity localized over nuclei (Fig. 3). DNA synthesis was also demonstrated by incubating cells with bromodeoxyuridine (BrdU), an analogue of thymidine which is incorporated into newly synthesized DNA (24). BrdU-labeled cells were detected using immunofluorescence with a monoclonal antibody to BrdU. Following exposure of cells after overnight culture to 10 μM BrdU for 3 h, 5–10% of the cells were labeled. When the exposure time was increased to 18 h, 47±8% (mean±SE, n = 4) of the cells has entered S phase, as evident by BrdU-labeled nuclei. This value was similar to that found in the studies using autoradiography.

Quantification of cell replication. Quantitative studies of cell replication used cell number, the activity of hexosaminidase, a lysosomal enzyme marker of cell mass, and the incorporation of [^{3}H]-THM into DNA, detected as TCA-precipitated radioactivity.

Stimulation of proliferation

Mitogenic response to serum. Central to this study is the response of epithelial replicating cells to mitogenic stimuli. Calf serum induced a dose-dependent increase in cell number, cell mass, and [^{3}H]-THM incorporation (Fig. 4, *A* and *B*). These patterns of response were similar; we found [^{3}H]-THM incorporation to be useful as our routine measure of cell proliferation. The maximal response to serum, generally a twofold increase over basal, was observed at serum concentrations between 0.5 and 2.5%. A significant mitogenic effect was found at a serum concentration of 0.1%, indicating a sensitive growth response of these cells to serum. Dog serum, collected from a single animal and heat-inactivated (30 min, 56°C), also produced similar stimulation on growth (data not illustrated). Even in serum-free conditions (• in Fig. 4 *B*), cells remained viable and incorporated [^{3}H]-THM at a slow but definite rate.

Mitogenic response to EGF and TGFα. Human EGF at doses between 1 pM and 1 nM produced a dose-dependent increase in [^{3}H]-THM incorporation into acid-precipitated material (Fig. 5 *A*); these studies were done with the cells incubated in the serum-free R_0 medium. EGF produced a similar dose response curve against a background of 0.2% calf serum; the response was additive with serum and of a similar magnitude to that found in serum-free conditions (data not illustrated). Transforming growth factor alpha (TGFα), a structurally related peptide that acts in part or totally through receptors

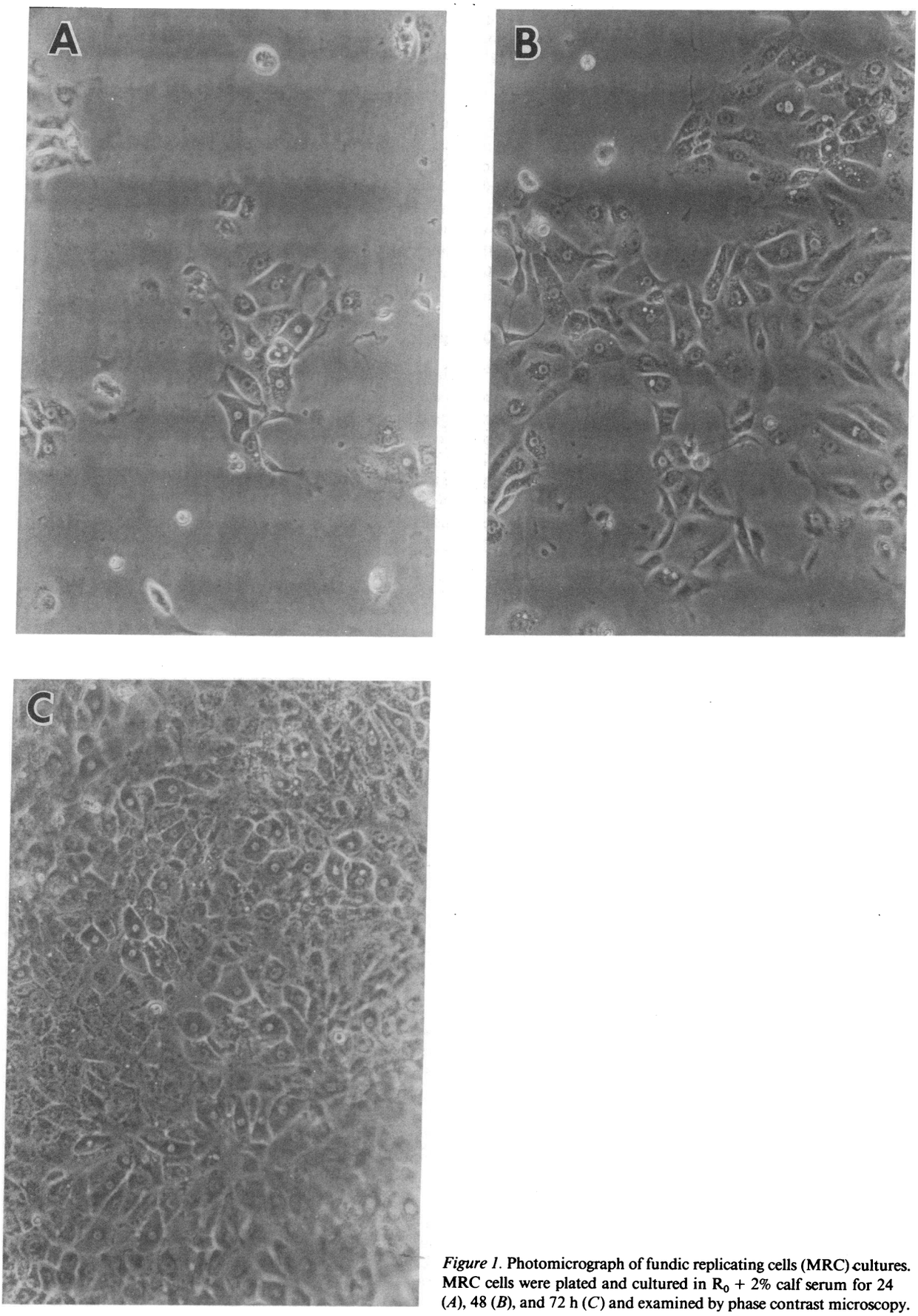

Figure 1. Photomicrograph of fundic replicating cells (MRC) cultures. MRC cells were plated and cultured in R_0 + 2% calf serum for 24 (*A*), 48 (*B*), and 72 h (*C*) and examined by phase contrast microscopy.

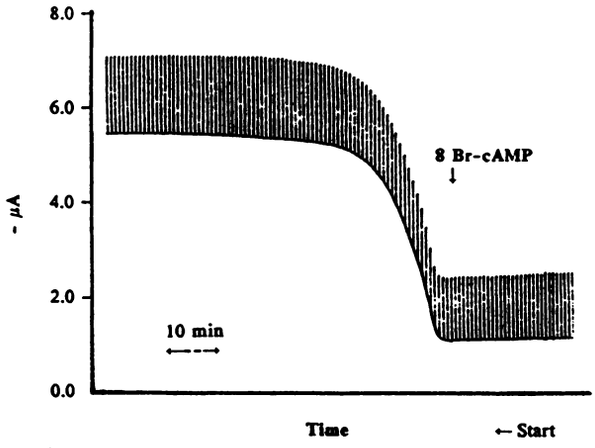

Figure 2. Polarized "transepithelial" function of MRC monolayers. MRC were cultured in R_0 + 2% calf serum on collagen-coated Millipore filters until a confluent monolayer had formed (72 h). Monolayers were mounted in Ussing chambers and electrophysiological properties studied. The solid line depicts the I_{sc} (short circuit current in microamperes). Conductance is depicted by the length of the deflection during a 5-μA pulse. From these data resistance was calculated to be 1,500 ohm · cm^2. This tracing illustrates an increase in I_{sc} after basolateral treatment with 0.1 mM 8-bromo cyclic AMP. These data are from a single monolayer and are representative of five other preparations.

shared with EGF, stimulated [^{3}H]-THM incorporation by these epithelial cells over a concentration range similar to EGF (Fig. 5 *B*). The magnitude of response to these two peptides was also similar.

Mitogenic response to insulin and IGF-I. Insulin and IGF-I also increased [^{3}H]-THM incorporation into DNA by these epithelial cultures under serum-free conditions (Fig. 6). The dose response for insulin was found at concentrations between 10 nM and 1 μM, whereas IGF-I produced mitogenic effects at concentrations between 0.1 and 100 nM. The response to IGF-I was also tested against a background of 0.2% serum (data not illustrated), with the magnitude of the response and concentration range similar to those observed under the serum-free conditions illustrated in Fig. 6.

Discussion

Establishing culture methods for replicating normal epithelial cells has proved challenging, in part because "epithelial" cell cultures are often overgrown by fibroblasts, precluding interpretation of data regarding epithelial cell replication. Several lines of evidence indicate that cultures prepared by our present methods after 24 h consist largely, if not exclusively, of epithelial cells. The great majority of cells display immunoreactivity with antibody to cytokeratin, which is a specific marker of epithelial cells (18). Monolayers form by 72 h and display the

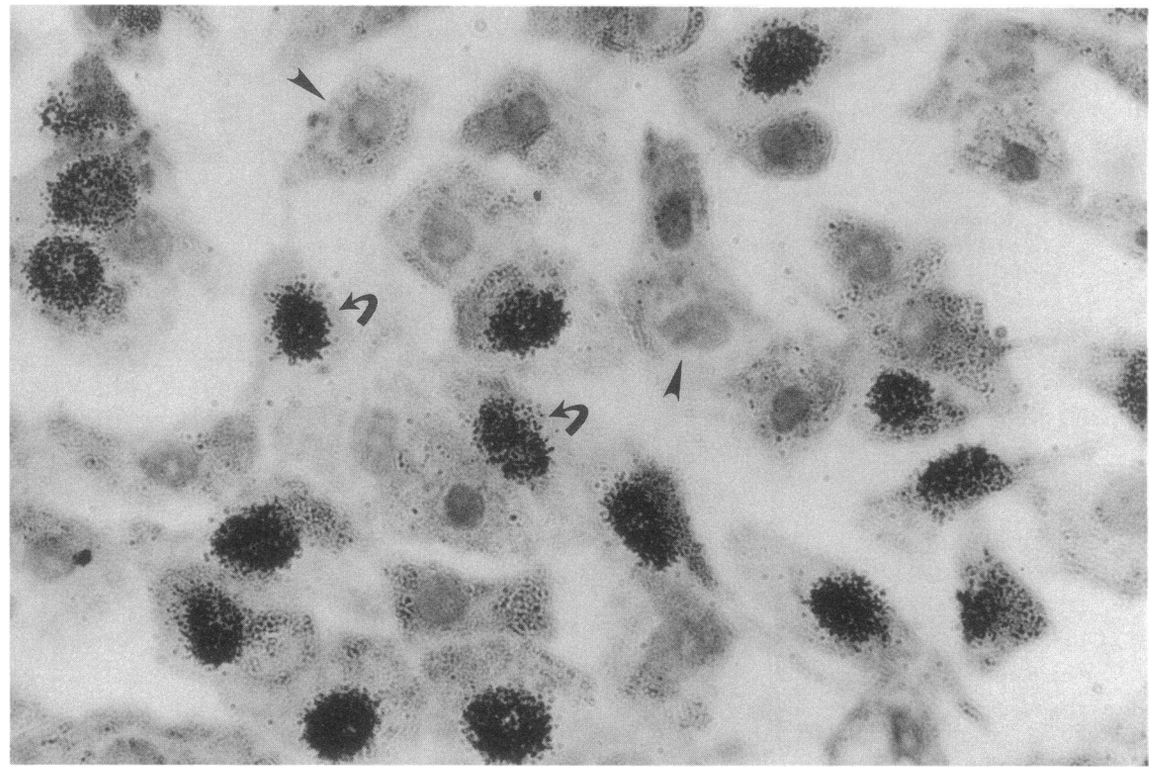

Figure 3. Cytokeratin in cultured gastric epithelial replicating cells. After overnight culture of MRC as outlined in the legend to Fig. 1, cells were washed and incubated with R_0 + 2% CS with 2 μCi/ml [^{3}H]-THM for an additional 24 h. Cultures were then washed with PBS and fixed in Bouin's. ABC staining with antibodies AE1/AE3 to cytokeratin was performed and followed by autoradiography. Slides were exposed for 3 wk before developing. Nuclei were counterstained by hematoxylin. Nuclei of cells that had synthesized DNA are peppered with grains (*arrow*), whereas nuclei of nonreplicating cells demonstrate only background radioactivity (*arrowhead*).

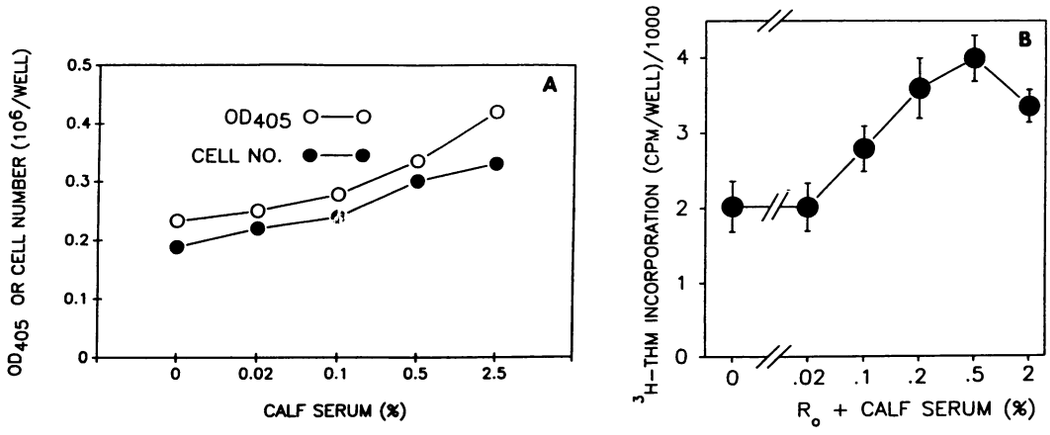

Figure 4. Serum effects on cell growth. Cells were cultured in R_0 with 2.0% calf serum overnight. The medium was then changed to the indicated concentrations of calf serum for another 72 h. (*A*) Cell growth was assessed by cell number, expressed as 10^6 cells per well, and cell mass, measured by hexosaminidase activity, expressed as the OD reading at 405 nm. (*B*) Cell growth was also determined by [^{3}H]-THM incorporation. Cells were cultured overnight, washed once, incubated in R_0 for 8 h and then placed in fresh R_0 in the absence and presence of the indicated concentrations of calf serum and 0.5 μCi/ml [^{3}H]-THM for 24 h. Cultures were washed with PBS and precipitated with 10% TCA. Acid precipitated counts were then solubilized in NaOH and counted. Data represent the mean±SE from four preparations and are expressed as radioactivity per well. Statistical significance was assessed using a paired *t* test.

electrophysiologic properties of a "tight" epithelium, with the mean resistance across these monolayers 1,600 ohm · cm^2. Polarized transport is established by finding a short circuit current response to stimulation by a cyclic AMP analogue; this I_{sc} is probably accounted for by absorption of Na^+ or secretion of Cl^- (15). Finding these characteristic features of a "tight epithelium" precludes the inclusion of nonepithelial cells in these monolayer cultures. Furthermore, using either autoradiography with cells incubated with [^{3}H]-THM or immunohistochemistry after incubation with the thymidine analogue BrdU, over half of the cells cultured in 2% serum synthesized DNA during an 18–24 h period. Coupling autoradiography for [^{3}H]-THM with immunohistochemistry for cytokeratin we found that the cells replicating in these cultures contained this epithelial marker. We therefore conclude that these cultures are composed of epithelial cells bound by tight junctions and that replicating epithelial cells are a predominant cellular component of these cultures.

Four indices were used to quantify cell replication, each indicating a steady increase in the number of cells over a 72-h period. (*a*) Phase contrast photomicrographs indicated cell replication, evident by an increase in the number of adherent cells. The number of adherent cells could increase without cell replication if delayed adherence of cells floating in the culture medium occurs; therefore, floating cells were removed after the initial overnight culture and before the start of these quantitative studies. (*b*) Counts of cells released from cultures by trypsin and calcium chelation confirmed an increase in cell number over time. (*c*) A hexosaminidase assay, which reflects cell mass, also indicated cell growth. However, use of this measure is limited by the dissociation that occurs between cell mass and cell number when confluence is approached. (*d*) These cultures incorporated [^{3}H]-THM into acid-precipitated material, which proved to be the most useful index of mitogenic response. Using these four measures, serum was found to induce proliferation of the fundic epithelial cells in these cultures. Although

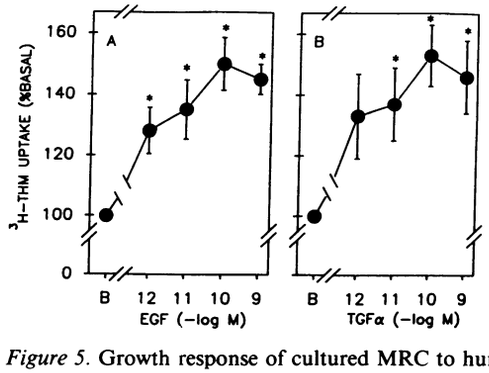

Figure 5. Growth response of cultured MRC to human EGF and TGFα. Cells were initially cultured as described in Fig. 4 *B*, and then placed in R_0 with the indicated concentrations of EGF (*A*) and TGFα (*B*). Cell growth was determined using [^{3}H]-THM incorporation. Data represent the mean±SE from four preparations and are expressed as the percentage of basal [^{3}H]-THM uptake. Statistical significance was assessed using a paired *t* test. *$P < 0.05$ vs. basal.

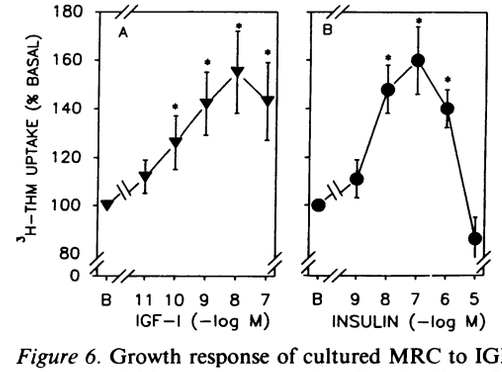

Figure 6. Growth response of cultured MRC to IGF-I and insulin. Cells were initially cultured as described in Fig. 4 *B*, after 8–12 h culturing in R_0, cells were then placed in R_0 with the indicated concentrations of IGF-I (*A*) and insulin (*B*). Cell growth was determined using [^{3}H]-THM uptake with treatment for 24 h. Data represent the mean±SE from four preparations and are expressed as percentage of basal [^{3}H]-THM uptake. Statistical significance was assessed using a paired *t* test. *$P < 0.05$ vs. basal.

> 50% of cultured epithelial cells were found to synthesize DNA during 48–72 h, our culture preparation was not homogeneous. This replicating cell population needs to be further characterized to determine whether these cells display markers of mucosal cell differentiation (e.g., pepsinogen I, H^+/K^+-ATPase, or mucin) at the time of division or subsequently express these markers in culture. Such studies are complex and require autoradiography coupled with immunohistochemistry.

Because our goal was to investigate the mechanisms regulating cell growth in the normal gastric mucosa, we sought to develop a primary culture system in which proliferative responses could be characterized within 72 h of removal from the intact tissue. Although some alteration in cell responses may occur upon dispersion or within hours of culture, our observations with this primary culture system will not be influenced by the alterations in cell characteristics that occur in transformed or nontransformed cell lines in prolonged culture. The doubling time of our fundic mucosal replicating cells in culture with serum is ~ 48–72 h, depending on culture conditions, and this compares favorably with estimates of 4–6 d in intact dog tissue (1). Thus, these fundic mucosal epithelial cells appear to replicate in culture at a rate roughly comparable to that found in vivo, suggesting that this system may serve as a good model for studying growth regulation of normal gastric mucosa.

Although the addition of serum is required for survival of many culture systems, its presence may perturb epithelial cell function and differentiation (25). Furthermore, we found that serum in concentrations as low as 0.1% induces proliferation of canine fundic epithelial cells, and its presence may thus confound interpretation of mitogenic responses to specific growth factors. However, fundic epithelial cells cultured by the present methods tolerate serum-free conditions and respond to at least two growth factors under these conditions. The initial adherence of cells was facilitated by adding 2% serum for the first 12 h of culture. However, after this initial period, serum could be removed without obvious deleterious effects on mitogenic responses. For some of present studies, we have inserted an 8–12-h wash step in serum-free and growth factor–free medium after the initial culture period to allow effects of residual serum to dissipate.

Cell growth is a highly regulated process; polypeptide growth factors acting on surface receptors are major modulating elements for growth control in proliferating epithelial and mesenchymal cells (26). EGF was the first growth factor acting in this fashion to be identified. EGF is synthesized in highest quantities by salivary glands, and it is also found in Bruenners glands from duodenum. EGF is therefore present in saliva and gastric juice, as well as serum. Luminal EGF has been reported to exert a mitogenic effect on gastric mucosa, but the physiologic importance of both luminal and circulating EGF remains uncertain. The study of EGF is further complicated by the existence of TGFα. TGFα also exerts a mitogenic effect in a variety of epithelial and mesenchymal tissues and in tumor cells. In contrast to EGF, which appears to be synthesized only in a few specific sites, TGFα is produced by many normal tissues, as well as by tumor cells (27, 28). TGFα message has been expressed in many tissues and cells, including the gastrointestinal tract (29), keratinocytes (28), and isolated rat intestinal cells (30). In canine gastric mucosa TGFα message appeared to be present in exocrine cells (31); however, a specific cellular localization was not established. Although the function of TGFα produced in normal tissues has not been established, our data are consistent with the hypothesis that this peptide exerts autocrine and/or paracrine growth control on fundic epithelial cells.

IGF-I, a peptide previously known as somatomedin C, mediates peripheral actions of growth hormone and is a potent mitogen in normal and neoplastic cells (32, 33). Many tissues and cell types produce IGF-I, including the adult gastrointestinal tract. In the intact rat, growth hormone induces growth of the fundic mucosa (2), although a specific role of IGF-I in regulating mucosal growth has not been elucidated. In our present study, we found that IGF-I and insulin stimulated [^{3}H]-THM incorporation by fundic epithelial cells. The greater potency of IGF-I compared to insulin suggests mediation by IGF-I receptors. Our data support the conclusion that IGF-I is a direct modulator of fundic epithelial cell growth, and are consistent with the hypothesis that IGF-I is an paracrine or autocrine mediator of gastrointestinal mucosal cell growth (32).

The ability of serum to promote cell growth in vitro probably reflects the presence of one or more specific factors, candidates include transferrin (34, 35), fibroblast growth factor (36), IGF-I and factors derived from platelets (37, 38). We found that the maximal responses to IGF-I and to EGF were additive with serum, possibly indicating that the effects of serum are not accounted for solely by either of these growth factors. Our present data only indicate that factors in serum potently induce mitogenesis in fundic mucosa; the identity and potential physiologic importance of the putative growth factors in serum remain to be established.

The present studies use a reductionist approach to study the regulation of growth in the fundic mucosa. This regulation is likely to be a complex process; in vivo hormonal mediators (gastrin) (2, 39) and neural pathways (40–42) stimulate fundic mucosal growth. Our present data support the hypothesis that TGFα and IGF-I, presumably acting by autocrine and/or paracrine mechanisms, also modulate growth in fundic mucosa. Other paracrine modulators could also be delivered from endothelial cells, fibroblasts, or macrophages. The presence of these numerous potential pathways and interactions complicates study of growth regulation in vivo, because these endogenous regulating factors may mask or interact with exogenous agents. Although such a reductionist system has the potential of defining the components of this complex regulation, there are several limitations. Although > 50% of the cells in these cultures are capable of dividing within a 24-h period, the cultures contain a heterogeneous cell population. The demonstration of a mitogenic response does not guarantee that the action is directly on the replicating cell. For example, our present data do not exclude the possibility that TGFα or IGF-I exert their growth stimulation via effects on neighboring cells mediated by release of a paracrine growth modulator. Further studies will be needed to localize EGF, TGFα, and IGF-I receptors to the replicating cells. Secondly, replicating epithelial cells may not represent a single population, but may include true stem cells (undifferentiated and uncommitted), as well as precursors which may be committed to and possibly express differentiation markers of a specific mucosal cell type. Lastly, although in vitro systems offer the potential of identifying specific cellular mechanisms exerting growth control, findings need to be related back to an intact, integrated system to determine which of

the candidate mechanisms are operative controlling growth under normal physiologic conditions or accelerating growth in response to acute injury or in response to chronic peptic ulceration.

Acknowledgments

The authors thank Dr. Martin Martin for discussions about these experiments and Deborah Avedian and Mary Tanner for their indispensable technical support. The authors also thank our collaborators, in particular Drs. Amram Ayalon, Marty Sanders, and Calvin Olson who helped in the development of methods for culturing mucosal cells. We also thank Drs. Catia Sternini, Nick Brecha, Che-Nan Chuang, and Karl Anderson for their ongoing help refining our skills in immunohistochemistry and autoradiography.

Supported by grants from the NIADDK AM 30444 and by the Medical Research Service of the Veterans Administration. Andrew Soll is a recipient of a Medical Investigatorship, VA Wadsworth Hospital Center.

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