Laminins and TGF-β maintain cell polarity and functionality of human gastric glandular epithelium

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Basque, Jean-René, Pierre Chailler, and Daniel Ménard. Laminins and TGF-β maintain cell polarity and functionality of human gastric glandular epithelium. Am J Physiol Cell Physiol 282: C873–C884, 2002. First published November 21, 2001; 10.1152/ajpcell.00150.2001.—The human gastric glandular epithelium produces a gastric lipase enzyme (HGL) that plays an important role in digestion of dietary triglycerides. To assess the involvement of extracellular matrix components and transforming growth factor-β1 (TGF-β1) in the regulation of this enzymic function, normal gastric epithelial cells were cultured on collagen type I, Matrigel, and laminins (LN)-1 and -2 with or without TGF-β1. Epithelial morphology and HGL expression were evaluated using microscopy techniques, enzymic assays, Western blot, Northern hybridization, and RT-PCR. A correlation was observed between the cell polarity status and the level of HGL expression. TGF-β1 alone or individual matrix components stimulated cell spreading and caused a downfall of HGL activity and mRNA. By contrast, Matrigel preserved the morphological features of differentiated epithelial cells and maintained HGL expression. The combination of LN’s with TGF-β1 (two constituents of Matrigel) exerted similar beneficial effects on epithelial cell polarity and evoked a 10-fold increase of HGL levels that was blunted by a neutralizing antibody against the α2-integrin subunit and by mitogen-activated protein kinase (MAPK) inhibitors PD-98059 (p42/p44) or SB-203580 (p38). This investigation demonstrates for the first time that a powerful synergism between a growth factor and basement membrane LNs positively influences cell polarity and functionality of the human gastric glandular epithelium through an activation of the α3β1-integrin and effectors of two MAPK pathways.

human stomach; extracellular matrix; integrin; mitogen-activated protein kinase

REGULATION AND MAINTENANCE of epithelial differentiation at the level of the gut mucosa is governed by extracellular signals present in the cell microenvironment. These are represented not only by growth factors but also by cell-to-cell interactions and their underlying extracellular matrix (ECM) (9, 32, 35). For epithelial cells, part of the ECM occurs in the form of a basement membrane that provides positional information and cues for cell polarity as well as signals that regulate cell behavior. Laminins (LNs) are among the major constituents of basement membranes, either native or reconstituted (Matrigel) (43), and they promote cellular adhesion, migration, proliferation, and expression of tissue-specific genes in differentiating epithelial cells and other tissues (13, 20, 33, 49). Furthermore, a number of growth factors including transforming growth factor-β1 (TGF-β1) are associated with basement membranes (50). The latter peptide regulates cell-matrix interactions (40), which implies that TGF-β1 may function as a regulator of epithelial morphogenesis and expression of tissue-specific proteins (21, 25, 39). In the context of gastric epithelial physiology, however, the combinatory effects of ECM components and TGF-β1 have never been specifically studied.

An original and important aspect of digestive functions attributed to the human gastric glandular epithelium pertains to hydrolysis of fat, which is assumed by a lipase enzyme produced by zymogenic chief cells (14, 30). We have shown that human gastric lipase (HGL) is expressed early during ontogeny (before midgestation) (31) and is uniquely colocalized with fundic-type peptisinogen (Pg5) in fetal chief cells (6). Our studies also revealed different regulatory patterns for HGL and Pg5 during in utero development and in culture experiments. Therefore, it appears essential to clearly understand the molecular mechanisms controlling the acquisition and maintenance of chief cell functions when one considers the physiological importance of gastric lipolysis and the fact that its role increases in the context of perinatal physiology (23) and pathological conditions (1) associated with pancreatic immaturity or insufficiency. For this purpose, adequate cellular models must be made available, and a promising tool for identifying the specific regulators of gastric digestive functions and their underlying mechanisms of action is a novel primary culture model representative of the normal human gastric epithelium (5). These cultures form coherent monolayers in the absence of a biological matrix, mesenchymal constituents, and hor-
mones. They contain all epithelial cell types, including functional glandular chief cells that retain their ability to produce digestive enzymes. Under such minimal conditions, however, the epithelial phenotype is altered, and the expression of HGL is spontaneously downregulated (5). Our new model thus appears unique to determine the appropriate extracellular environment necessary for such complex regulatory events as the maintenance of epithelial cell polarity and the induction of gastric zymogen expression.

On a functional basis, growth and differentiation of gut epithelial cells likely depend on their interaction with ECM components using cell-surface receptors of the integrin family (9, 10). The α5β1-integrin, which is unique among these receptors for having a dual specificity for collagen and LNs, has been shown to play a key role in mammary epithelial morphogenesis in vitro (12, 27). We recently analyzed the distribution of ECM proteins and receptors in developing human stomach (16, 46) and provided evidence for the potential involvement of LN-1 (or the closely related LN-10), LN-2, and α5β1-integrin in epithelial glandular differentiation. In the current investigation, we have demonstrated that collagen type I and Matrigel, respectively, exert negative and positive effects on cell polarity and HGL expression in primary cultures of gastric epithelium. In fact, the maintenance of epithelial morphology and functionality of cultured cells is mediated by a unique to determine the appropriate extracellular environment necessary for such complex regulatory processes as the maintenance of epithelial cell polarity.

MATERIALS AND METHODS

Specimens. Tissues from 47 fetuses varying in age from 17 to 20 wk of gestation postfertilization were obtained from normal elective pregnancy terminations (41). No tissues were collected from cases associated with known fetal abnormality or fetal death. Studies were approved by the Institutional Human Subject Review Board. The stomach was brought to the culture room, immersed in dissection medium, i.e., Leibovitz L-15 (GIBCO BRL/Life Technologies, Burlington, ON, Canada) plus gentamicin and nystatin (40 μg/ml each), and prepared within 30 min at room temperature.

Primary culture. Cardia and pyloric antrum were excised from the stomach, leaving the body and fundus regions. Tissues were then cut into explants (3 × 3 mm2) and rinsed with dissection medium. The gastric epithelium was isolated with dissection medium. The gastric epithelium was isolated from the stomach, leaving the body and fundus regions.

Microscopy and immunocytochemistry. Tissue sections (3-μm-thick) were fixed for 12 min in 3% formaldehyde or 5 min in acetone:chloroform (1:1) at 4°C and then permeabilized with 0.1% Triton X-100 for 3 min and incubated with primary antibodies directed against α5- and α3-integrin sub-units (clones P1E6 and P1B5, respectively; GIBCO) were added after initial attachment (6 h) at the 10 μg/ml concentration.

As primary antibodies we used secondary antibodies (1:30 and 1:40, respectively) and were added for 45 min. Specimens mounted in glycerol/PBS were observed using a Reichert Polvar 2 microscope (Leica Canada, St-Laurent, QC, Canada) equipped for epifluorescence. All appropriate controls where the primary antibody was omitted were performed and shown negative, as exemplified in Fig. 7. For ultrastructural studies, specimens were prefixed for 15 min at room temperature in an equal volume of culture medium and fresh 2.8% glutaraldehyde diluted in 0.2 M cacodylate buffer containing 7.5% ultrapure sucrose, fixed for 30 min in 2.8% glutaraldehyde-7.5% sucrose diluted in 0.1 M cacodylate, and postfixed in 2% osmium tetroxide diluted in 0.1 M cacodylate for 1 h. They were dehydrated and covered with a 3-mm layer of Epon 812 resin. After polymerization (48 h at 60°C), the plastic substratum was detached and specimens were inventoried and reembedded. Thin sections were visualized on a JEM-100CX electron microscope (JEOL, Peabody, MA).

Gel electrophoresis and immunoblotting. Total protein samples from three independent experiments were lysed in 2× buffer containing 2% β-mercaptoethanol in 20 mmol Tris-HCl (pH 6.8). As standardized earlier for gastric primary cultures (5), 60- to 80-μg aliquots were resolved by SDS-PAGE on 12% acrylamide gels, transferred onto nitrocellulose membranes, and processed with a Western-Light Plus chemiluminescence detection system (Tropix, Bedford, MA). After initial quenching in blocking buffer (1% Blotto-PBS), the following specific primary antibodies were added: anti-E-cadherin (1:1,000), anti-α2-integrin subunit (1:400), and anti-cytokeratin-18 (1:5,000, clone CY-90; Sigma-Aldrich, Oakville, ON, Canada). After being washed in PBS, membranes were incubated with biotinylated goat anti-rab-
bit or anti-mouse immunoglobulin G (1:10,000) and with alkaline phosphatase-conjugated streptavidin (1:20,000). Finally, immunoreactive proteins were revealed with a solution of ultrapure CSPD (Tropix) containing Nitro-Block (1:20). Autoradiographs were exposed in a linear range by using a LKB XL Ultrascan (Pharmacia, Baie d’Urfe, QC, Canada).

Northern hybridization and reverse transcription-polymerase chain reaction. Total RNA was isolated from three separate primary cultures by using TriPure reagent (Boehringer Mannheim) and processed as formerly described (5, 44). For Northern blot analysis, equivalent amounts of RNA (20 μg) were separated by electrophoresis through 1% agarose gels containing 6% formaldehyde and blotted onto nylon membranes (Hybond-N; Amersham, Oakville, ON, Canada). Equal loading was confirmed by ethidium bromide staining. Membranes were hybridized with cDNA probes specific for HGL and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5, 44), blotted dry, and autoradiographed.

The reverse transcription (RT) reaction was carried out at 37°C by using Ready-To-Go First-Strand Beads (Amersham) to which was added 3 μg of total RNA mixed with oligo(dT)12–18 and SuperScript RT RNase H− reverse transcriptase. The cDNA samples prepared were directly used for polymerase chain reaction (PCR) amplification. PCR primers complementary to the reported cDNA sequences of HGL and GAPDH were used: 100 pmol of both upstream sense primers LIPASE-1, 5’-CTGGAGGAACGTGAGTCCA-3’, and GAPDH-1, 5’-CCACCATGGCAAATTCCATGGCA-3’, as well as 100 pmol of downstream antisense primers LIPASE-2, 5’-AGAAGCAGTCAATTGT-3’, and GAPDH-2, 5’-CTTACAAGGCAAGTGTCAGTGTACC-3’, all in the presence of 2.5 units of Taq polymerase (Roche Molecular Biochemicals, Laval, QC, Canada). The cDNA was subjected to 25 cycles of denaturation (1 min at 94°C), annealing (1 min at 52–63°C), and primer extension (1 min at 72°C) in a DNA thermal cycler (model 480; Perkin Elmer Applied Biosystems, Mississauga, ON, Canada). Amplification yielded a 1,311-base pair (bp) fragment for HGL and a 612-bp fragment for GAPDH.

Enzymic assays: lipase and pepsin. Lipolytic activity was measured by using a long-chain triglyceride substrate (tri[1-14C]oleic acid; Amersham) and fatty acid-free BSA (A-6003; Sigma-Aldrich) as carriers of released fatty acids, and the emulsion was prepared by sonication as described previously (31). The assay system contained the following reagents in a final volume of 200 μl: 1.2 μmol of labeled triglyceride, 10 μmol of citrate-phosphate buffer (pH 6.0), 0.1 μmol of BSA, 2 μmol of Triton X-100, and 100 μl of cell homogenate. Free [14C]oleic acid produced after a 1-h incubation was separated by liquid-liquid partition in chloroform-methanol-heptane (1:41:1:25:1), precipitated with 0.1 M carbonate-borate buffer (pH 10.5), and quantitated by liquid scintillation spectrometry (11). Specific lipase activity was expressed as nanomoles of free fatty acid released per minute per milligram of protein. Pepsin activity resulting from the activation of pepsinogen at acid pH was measured in 100 μl of cell homogenate by adding 1 ml of 2% (wt/vol) diazoyed hemoglobin (Sigma-Aldrich) diluted in 0.1 M glycine-HCl buffer (pH 3.0). The reaction was carried out at 37°C for 10 min and stopped with 6.2% (wt/vol) trichloroacetic acid. The resulting free amino acid products were separated by centrifugation and quantitated by spectrometry using an L-tyrosine standard. Specific pepsin activity was expressed in international units (μmol/min) per milligram of protein. Protein content of the homogenates was measured using Folin reagent. Results are reported as means ± SE, and the statistical significance of differences in enzyme activity between culture intervals or treatments was established at 95% and determined by analysis of variance followed by Student’s t-test when significance was indicated (number of experiments is specified in legends of Figs. 1–8).

RESULTS

Epithelial cell morphology. Freshly isolated gastric epithelium was resuspended in culture fluid and then fragmented mechanically into multicellular aggregates, and this material was plated in dishes precoated with collagen type I or Matrigel. Primary cultures seeded on collagen type I attached and spread rapidly, as visualized after 48 h of culture (Fig. 1A). Between this interval and day 3, they had already formed a confluent monolayer. In the presence of Matrigel, epithelial suspensions also attached rapidly but, rather, formed compact multicellular structures or organoids (Fig. 1, B and C). We next sought to identify the specific constituents of Matrigel matrix responsible for this striking morphological feature. When incubated with purified LN-1, LN-2, LN-1/LN-2 mixture, or 5 ng/ml TGF-β1, epithelial cells attached and spread rapidly (Fig. 1, D and E), recalling their behavior on collagen type I. Interestingly, the use of LN-1 and/or LN-2 in combination with TGF-β1 allowed the formation of compact epithelial cell colonies after 48 h of culture (Fig. 1P). LNs and TGF-β1 thus synergized in the present system to reproduce the beneficial effect of Matrigel.

When viewed by transmission electron microscopy after 48 h, gastric epithelial cells exhibited a different morphology depending on the biological matrix used as substrate. Cells cultured on collagen type I were generally characterized by a relatively less polarized phenotype (Fig. 2A). They grew as flat monolayers displaying small and sparse microvilli on their apical surfaces, and few secretory granules were observed. On the contrary, addition of Matrigel resulted in the presentation of three-dimensional cellular networks with well-polarized epithelial cells (Fig. 2, B and C). Their integrity and polarity were indicated by the adequate intracellular localization of basal nuclei, well-organized junctional complexes at the apical margins of cells, and abundant secretory organelles. However, gastric epithelial cells cultured with purified LN-1, LN-2, or TGF-β1 alone always exhibited a less polarized phenotype (Fig. 2A). They grew as flat monolayers displaying small and sparse microvilli on their apical surfaces, and few secretory granules were observed. On the contrary, addition of Matrigel resulted in the presentation of three-dimensional cellular networks with well-polarized epithelial cells (Fig. 2, B and C). Their integrity and polarity were indicated by the adequate intracellular localization of basal nuclei, well-organized junctional complexes at the apical margins of cells, and abundant secretory organelles. However, gastric epithelial cells cultured with purified LN-1, LN-2, or TGF-β1 alone always exhibited a less polarized appearance (Fig. 2, D and E), as observed with collagen type I. The combination of TGF-β1 with LN-1, LN-2, or LN-1/LN-2 mixture (Fig. 2F) exerted the same beneficial effects as Matrigel on the maintenance of differentiated phenotypes and cell polarity in epithelial cells. Cultured cells displayed a basal nucleus as well as specialized junctions, numerous microvilli, and secretory granules in their apical regions.

Gastric lipase expression in zymogenic chief cells. To understand the molecular mechanisms involved in the maintenance of human chief cell functional differentiation, we next verified how HGL expression would be modulated in the same culture conditions. First, the use of collagen type I coating induced a very significant decrease of HGL activity (Fig. 3A), and this effect correlated with a drastic reduction of HGL mRNA...
signals during the incubation period (Fig. 3B). The level of immunoreactive E-cadherin protein also diminished markedly between 1.5 and 5 days of culture (Fig. 3C). By contrast, Matrigel prevented this downregulation, because cellular lipase activity (Fig. 4A) and HGL mRNA levels (Fig. 4B) remained constant during culture. Of note, HGL activity levels measured after 1.5 days in the presence of Matrigel were fourfold higher than those reported on collagen type I. Matrigel also maintained the expression of E-cadherin protein (Fig. 4C) and its adequate distribution at sites of cell-to-cell contact in multicellular polarized clusters after 1.5 (Fig. 4D) and 5 days of culture (Fig. 4E).

To delineate the contribution of individual Matrigel components affecting HGL gene expression in our primary culture model, we investigated the potential implication of LN-1, LN-2, and TGF-β1. Seeding on both purified LNs resulted in a decrease of HGL activity and mRNA levels during culture (Fig. 3A and D). Compared with collagen type I however, HGL activity was significantly higher at each time interval, and the expression of E-cadherin protein remained constant (not shown), although its subcellular localization was altered. After 5 days, the immunoreactive staining appeared cytoplasmic and diffuse in some cultured cells, indicating that E-cadherin was dissociated from intercellular contacts (Fig. 3E). Of note, each purified LN used separately gave similar results (not shown). TGF-β1 alone did not prevent the downfall of HGL cellular activity (Fig. 3A) and mRNA levels during the same culture interval. Most importantly, the combination of TGF-β1 with LN-1, LN-2, or LN-1/LN-2 (Fig. 5) induced a progressive increase of HGL activity (Fig. 5A) and mRNA levels (Fig. 5B); compared with Matrigel and collagen type I, lipase activity was significantly upregulated 2.4- and 10-fold after 1.5 days. LNs/TGF-β1 also maintained constant E-cadherin protein levels (Fig. 5C) and preserved its adequate localization at cell-cell boundaries in epithelial clusters after 1.5 (Fig. 5D) and 5 days of culture (Fig. 5E). Chief cells present in these compact and well-polarized structures showed a granule-like distribution of immunoreactive HGL at 1.5 days postplating (Fig. 6A), and staining was most intense after 5 days (Fig. 6B). Ultrastructural analyses also revealed highly polarized secretory cells reminiscent of those found in gastric glands. A significant proportion contained supranuclear secretory vesicles with a heterogenous core (Fig. 6C) characteristic of the zymogen granules found in fetal chief cells (6). Junctional complexes (Fig. 6D) were visual-

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**Fig. 1.** Optical microscopy of gastric epithelial primary cultures. By 48 h postplating, cell colonies cultured on collagen type I had spread intensely (A). In the presence of Matrigel, compact multicellular clusters (arrows) were visible at the center of epithelial colonies (B and C). When seeded on purified laminin (LN)-1 (D), LN-2, or transforming growth factor (TGF)-β1 alone (E), aggregates spread and eventually generated monolayers of flattened cells, as shown with collagen type I. Combination of LNs (LN1/LN2) with TGF-β1 (F) caused the formation of dense multicellular structures (arrow) similar to those noted on Matrigel. Bar, 50 μm (phase microscopy, except B under bright field).
Fig. 2. Transmission electron microscopy of Epon-embedded gastric primary cultures after 48 h. On collagen type I (A), cells grew as coherent flat monolayers displaying sparse microvilli on their apical membrane (arrows). Matrigel maintained epithelial colonies as compact organoids where secretory cells were sometimes facing a central lumen (B). Individual cells displayed a polarized phenotype and abundant granules in their supranuclear cytoplasm (C). Gastric cells cultured on LN-1, LN-2 (D), or TGF-β1 alone (E) exhibited a depolarized appearance and few electron-luscent granules (asterisks). In the combined presence of TGF-β1 and LNIs (LN1/LN2 mixture is shown) (F), epithelial cells in multicellular aggregates were characterized by intercellular junctions (open arrowheads), apical microvilli, basal nuclei, and secretory granules. Bar, 1 μm.
ized at apical margins between cells as indicated by the presence of zonula occludens (tight junction), zonula adherens (adherens junction), and macula adherens (desmosome). Finally, it is noteworthy that pepsin activity resulting from the activation of cellular pepsinogen (Pg5) was not up- or downmodulated with biological matrices, as opposed to HGL, except for a slight stimulation with Matrigel at the end of the incubation period (compared with all other conditions; see Table 1).

Involvement of α2-integrin in the maintenance of HGL expression. The recent observation that α2β1-integrin expression may be relevant to the differentiation and/or maintenance of human glandular chief cells (16) prompted us to verify its presence in gastric primary cultures seeded on LNs with TGF-β1. Using the P1E6 monoclonal antibody that reacts with nonoverlapping epitopes on α2-subunit, we observed immunoreactivity at cell-cell boundaries during the initiation of culture (Fig. 7A). After 5 days postplating, the α2-subunit was properly localized at the periphery of cells (Fig. 7B), and Western blotting experiments confirmed the continuous expression of the α2-subunit from 1.5 days onward (Fig. 7D). To assess the possible role of α2β1-integrin in the upregulation of HGL expression, we performed neutralization assays with the same antibody. After initial attachment (6 h) of epithelial cells on LNs/TGF-β1, addition of 10 μg/ml anti-α2 for 48 h produced a significant reduction of HGL mRNA levels in culture, as revealed by RT-PCR experiments (Fig. 7E, left). In another set of experiments, addition of a neutralizing antibody against the α3-integrin subunit, poorly expressed in gastric glandular cells (16) as well as the addition of a nonrelevant anti-keratin-18 immunoglobulin did not modulate HGL (Fig. 7E, right), thus confirming the specificity of response to anti-α2 antibody.

Implication of MAPK cascades in HGL expression. To determine whether intracellular MAPK cascades associated with growth factor and ECM signaling pathways...
take part in the LNs/TGF-β1-induced regulation of HGL expression in chief cells, we tested selective inhibitors of the MKK-1/2 p42/p44 pathway (PD-98059) and p38/β1/β2 (SB-203580). Compared with gastric primary cultures showing maximal levels of HGL activity with LNs/TGF-β1, addition of PD-98059 or SB-203580 caused a significant decrease (Fig. 8A) without affecting pepsin activity (Table 1). RT-PCR experiments also were performed after 48 h of culture, and HGL mRNA signals were analyzed by semiquantitative densitometry (Fig. 8B). As expected, each compound induced a strong downregulation of HGL mRNA levels (~50% with PD-98059 and ~80% with SB-203580 vs. LNs/TGF-β1 without inhibitors). More interestingly, the simultaneous presence of both inhibitors (PD-98059 + SB-203580) completely abolished mRNA expression, thus suggesting that the synergistic response to LNs/TGF-β1 involves a parallel stimulation of p42/p44 and p38 MAPK pathways. Their effects seem to be mediated directly through MAPK function, because PD-98059 and SB-203580 did not modulate the expression of α2-integrin subunit protein (Fig. 8C) and because we observed no significant alteration of the cell phenotype during the 48-h incubation period (data not shown).

DISCUSSION

In recent years, an understanding of the intrinsic mechanisms by which mucosal growth factors and ECM cooperatively regulate gut epithelial functions has emerged. One current hypothesis is that paracrine and autocrine growth factors mainly contribute to the stimulation of cell proliferation and migration, often leading to expansion of the mucigenic cell lineage,
while repressing the terminal differentiation of digestive cells (32, 35). We have demonstrated that members of the epidermal growth factor (EGF) family stimulate the proliferation of epithelial precursors and the synthesis of mucus and that they downregulate the expression of HGL in maturing chief cells (44, 47), supporting the suggestion that this concept would apply to the human stomach. According to another recent hypothesis, the spatiotemporal expression of ECM components, and basement membrane proteins in particular, may play an inductive or permissive role during the morphogenesis of digestive functional units and their maintenance in the mature gastrointestinal tract (9, 10).

The present study was conducted to investigate the contribution of ECM on the maintenance of epithelial morphology and digestive functions in isolated human gastric cells. To decipher the cellular and molecular mechanisms involved, we developed a new primary culture model that is free of mesenchymal/submucosal influences and that is representative of the intact fetal gastric epithelium (5, 6). The model allows the growth and maintenance of all gastric epithelial cell types, including functional glandular chief cells, in the absence of added biological matrix. No signs of cytoplasmic vacuolization and cell detachment are visible during the first week of culture. Under these minimal conditions, however, attachment to the culture substratum is effective after 1 day, the polarity status of epithelial cells is modified, and the expression of HGL in chief cells is spontaneously downregulated (5). Logically, one could suggest that these phenomena could be attributed to the lack of ECM constituents, as reported for other epithelial cell types (4, 13).

Our experiments clearly demonstrate that collagen type I allowed the rapid attachment of epithelial aggregates that spread intensely and lost their polarized phenotype, while the expression of E-cadherin protein was downregulated. These effects are consistent with earlier observations made on cultures of intestinal epithelial cells where this interstitial ECM component optimizes cell adhesion and migratory behavior (7, 8). Furthermore, the expression of HGL in gastric cells was drastically repressed under these conditions. When Matrigel was used as coating, cell spreading was less stimulated and morphological parameters were all ameliorated. Epithelial aggregates adhered and conserved a compact organization in which individual cells retained the functional characteristics of a polarized gastric epithelium, as verified at the ultrastructural level and by the maintenance of E-cadherin in epithelial junctions. Interestingly, this reconstituted basement membrane exerted a beneficial effect on HGL gene expression as lipase activity and HGL mRNA levels remained constant. Such observations are in accordance with those reported for intestinal epithelial

Fig. 6. Combinatory effects of LNs (LN-1/LN-2 mixture) and TGF-β1 on HGL immunoreactivity and epithelial morphology. With the use of an anti-HGL antibody, chief cells inside dense and compact colonies showed a strong immunoreactivity after 1.5 days of culture (A). Staining was most intense after 5 days of culture (B). Ultrastructural analyses revealed numerous secretory vesicles (C) as well as junctional complexes at apical margins between cells (D) comprising zonula occludens (ZO), zonula adherens (ZA), and macula adherens (MA). Bars, 50 μm (A and B); 0.5 μm (C and D).
A logical follow-up to these experiments was to investigate the implication of LN-1 and TGF-β1 in the Matrigel-induced response, because it has been shown that they are major constituents of this reconstituted basement membrane (42, 50). Although the regulatory potential of LN-1 on the expression of brush-border enzymes in intestinal cells has been verified by a number of investigators (8, 22, 36, 48), we compared herein the effects of two LN variants on the basis of the differential distribution of LN-1/LN-10 (ubiquitous) and LN-2 (base of gland) along the gastric foveolus-gland axis (16, 46). Also, the TGF-β1 peptide exerts a biological activity different from other growth factors, because it generally acts as an antimitogen on cultured epithelial cells and sometimes promotes their morphological and functional differentiation (40). Our data indicate that gastric epithelial cells seeded on LN-1, LN-2, or LN-1/LN-2 mixture or in the presence of TGF-β1 alone exhibited higher HGL activity compared with collagen type I, which downregulated HGL more drastically after 5 days. This variation may be related

cells (15, 22, 36) and other cell types (2, 4, 37), illustrating that Matrigel is particularly effective as a culture substratum for maintaining cell polarity or even eliciting a spectrum of phenotypic changes toward full differentiation. Also of note, pepsin enzymic activity was measured in primary gastric epithelial cultures: effect of biological matrices

Table 1. Specific pepsin activity measured in primary gastric epithelial cultures: effect of biological matrices and MAPK inhibitors

<table>
<thead>
<tr>
<th>Condition</th>
<th>Days of Culture</th>
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<tr>
<td></td>
<td>1.5</td>
<td>3</td>
<td>5</td>
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<tr>
<td>Collagen I</td>
<td>100 ± 9</td>
<td>122 ± 11</td>
<td>111 ± 7</td>
</tr>
<tr>
<td>Matrigel</td>
<td>130 ± 15</td>
<td>140 ± 6</td>
<td>150 ± 17*a</td>
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<tr>
<td>LN-1/LN-2</td>
<td>97 ± 4</td>
<td>90 ± 12</td>
<td>87 ± 11</td>
</tr>
<tr>
<td>TGF-β1 alone</td>
<td>107 ± 12</td>
<td>102 ± 10</td>
<td>91 ± 7</td>
</tr>
<tr>
<td>LN/TGF-β1</td>
<td>104 ± 9</td>
<td>109 ± 11</td>
<td>99 ± 14</td>
</tr>
<tr>
<td>LN/TGF-β1 + PD</td>
<td>98 ± 3</td>
<td>105 ± 9</td>
<td>n.d.</td>
</tr>
<tr>
<td>LN/TGF-β1 + SB</td>
<td>100 ± 9</td>
<td>91 ± 13</td>
<td>n.d.</td>
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Values are in IU/mg protein and represent means ± SE of 3–6 separate experiments. LN-1, LN-2, and LN-1/LN-2 mixture gave similar results and the latter is shown. LN, laminin; TGF-β1, transforming growth factor-β1; PD, PD-98059; SB, SB-203580. *Significant difference between cellular activities on Matrigel and other conditions after 5 days (P < 0.05); n.d., not determined.

Fig. 7. Role of α2-integrin subunit in the maintenance of HGL expression. In 3 different cultures seeded on LN-1/LN-2 with TGF-β1, immunodetection with the anti-α2 P1E6 antibody revealed staining at cell-cell boundaries after 1.5 (A) and 5 days (B). Controls for which the primary antibody was omitted showed no significant staining (C). D: representative Western blot analysis at 1.5, 3, and 5 days showing α2-integrin protein and K18 control. E: 2 representative RT-PCR for HGL mRNA in cells cultured 48 h on LNs/TGF-β1 in the presence of neutralizing antibodies: anti-α2-integrin (+α2; inhibitory effect), anti-α3-integrin (+α3; no effect), and a nonrelevant anti-K18 immunoglobulin (+IgG; no effect). GAPDH mRNA probe was used as an internal control. Bar, 50 μm.

Fig. 8. Role of mitogen-activated protein kinase (MAPK) pathways in induction of HGL expression. A: specific HGL activity measured in cells after 48 h of culture in the presence of selective inhibitors of p42/44 [PD-98059 (PD), 20 μM] and p38α/β [SB-203580 (SB), 20 μM] MAPK pathways. Values represent means ± SE of 3 independent experiments. *Significant decrease vs. LN/TGF-β1 (P < 0.05). B: RT-PCR for HGL mRNA and GAPDH control in 3 cultures maintained 48 h on LNs/TGF-β1 with PD and/or SB. C: Western blot analysis of α2-integrin subunit protein and K18 control shows no alteration in the presence of inhibitors.

AJP-Cell Physiol • VOL 282 • APRIL 2002 • www.ajpcell.org
to the differential regulation of E-cadherin, because only collagen type I strongly repressed this junctional marker during the first days of incubation. However, cells maintained with LNs or TGF-β1 formed a flattened monolayer, and a progressive and significant downregulation of HGL still occurred. In the same cultures, signs of E-cadherin dissociation from cell-cell contacts were observed in the long term. In comparison, when LNs and TGF-β1 were added simultaneously, the primary cultures were composed of isolated and compact organoid structures and the highly polarized phenotype of epithelial cells was preserved. These conditions also maintained a proper distribution of E-cadherin at cell-cell boundaries and the presence of a functional secretory apparatus in chief cells. LNs/TGF-β1 even upregulated HGL levels compared with Matrigel, possibly reflecting the purified nature of the ligands. We also noted that each LN form as well as the LN-1/LN-2 mixture exerted similar effects, suggesting that any possible difference in their secondary or tertiary structure has no significant repercussion on LN bioactivity in our system, at least on cell morphology and HGL. In summary, the last results indicate that gastric primary cultures necessarily require both LN and TGF-β1 supplements for expressing their optimal functional characteristics, because only this combination is able to maintain epithelial cell polarity and induce HGL expression. Moreover, such findings suggest that the regulation of gastric digestive functions would be coordinated by a delicate balance of inducer and repressor factors. In our basic reference describing the primary culture methodology (5), we reported that EGF negatively regulates HGL mRNA and activity without affecting pepsin, as previously demonstrated in gastric mucosa explants for EGF itself and other mitogens like TGF-α, insulin-like growth factor (IGF)-I, and IGF-II (44, 45).

ECM components such as basement membrane LNs modulate gut epithelial cell functions by interacting with integrin receptors (9, 10). Our previous study (16) revealed an increased expression and redistribution of α2-integrin subunit at the basal membrane of epithelial cells in the inferior portion of the fetal gastric gland. This finding suggested that the α2β1-integrin may play a key role in the differentiation and/or maintenance of glandular cell types, especially zymogenic chief cells vs. the parietal cell lineage where expression of α2-subunit was strongly repressed. This view is supported by the fact that simultaneous addition of a neutralizing antibody against the α2-subunit with LNs/TGF-β1 almost abrogated HGL mRNA production in primary cultures after 48 h. In addition, there is increasing evidence that intracellular signals elicited by hormones, growth factors, and integrin ligands exert their cooperativity on cell responses through a convergent regulation of MAPK cascades (3, 17). Several investigators have indeed demonstrated that the presence of LNs or TGF-β commonly elicits the activation of p42/p44 MAPKs (18, 24, 38). This was specifically verified for intestinal cell lines (29, 52), whereas TGF-β also activates p38α/β MAPKs in gastric cell cultures (26). Further adding to the relevance of MAPK function in gastric physiology, we have shown that an acute deregulation of basal p42/p44 activity by the mitogens EGF and TGF-α was partly responsible for inhibiting HGL expression (44). As shown here, the specific inhibitor of p42/p44 cascade, PD-98059 (19), significantly reduced the stimulatory effect of LNs/TGF-β1 on HGL activity and mRNA after 48 h, and this downregulation was even stronger with SB-203580, an inhibitor of p38 (28). Also, their combination completely abrogated the inductive effect of LNs/TGF-β1. Therefore, our results suggest that a parallel activation of two MAPK cascades is necessary for an optimal regulation of HGL gene expression in chief cells. Such cooperativity has been observed in myoblasts where both p38 and p42/p44 activities are required to activate myogenic transcription and hypertrophic growth of myotubes (51). In conclusion, the present investigation identifies TGF-β1 and LNs as positive regulators of cell polarity and HGL expression in gastric epithelial cultures. It is thus the first demonstration that a powerful synergism between a growth factor and basement membrane LNs maintains the functionality of human gastric glandular epithelial cells, including the zymogenic chief cell population, through an activation of the α2β1-integrin and effectors of two MAPK pathways.

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AJP-Cell Physiol • VOL 282 • APRIL 2002 • www.ajpcell.org

AJP-Cell Physiol • VOL. 282 • APRIL 2002 • www.ajpcell.org
