Hepatocyte growth factor regulates the development of highly pure cultured chief cells from rat stomach by stimulating chief cell proliferation in vitro

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Tashima K, Zhang S, Ragasa R, Nakamura E, Seo JH, Muvaffak A, Hagen SJ. Hepatocyte growth factor regulates the development of highly pure cultured chief cells from rat stomach by stimulating chief cell proliferation in vitro. Am J Physiol Gastrointest Liver Physiol 296: G319–G329, 2009. First published November 20, 2008; doi:10.1152/ajpgi.90355.2008.—The physiology of gastric epithelial cells is often studied by using cancer cell lines, which may or may not provide information relevant to normal cells. Because few models exist to study chief cell physiology in vitro, our purpose was to develop primary cultured chief cells from rodent species that are structurally and functionally similar to native chief cells. For this, isolated chief cells from the rat stomach, purified by counterflow elutriation and density gradient centrifugation, were grown in media with growth factors. Purity and the continuity of tight junctions were determined, and permeability, viability, transepithelial resistance (TER), cell number and proliferation, and pepsinogen secretion in response to carbachol were measured. When plated in media alone or with basic fibroblast growth factor, the isolated chief cells attached by 2 days and were confluent by 4 days after seeding. However, tight junctions were discontinuous, TER was less than 300 Ω·cm², and permeability was high. In contrast, chief cells incubated with hepatocyte growth factor (HGF) were confluent in 3 days and had a TER greater than 2,000 Ω·cm², continuous tight junctions, and low permeability. EGF was intermediate. HGF facilitated monolayer development by increasing cell number, which occurred by the proliferation of chief cells. Chief cell cultures, grown with HGF, consisted of more than 99% gastric intrinsic factor-expressing cells and showed robust pepsinogen secretion. Coexpression studies for neck and chief cell markers also show that the cultures are a mixture of mature, immature, and transitional zone cells. This model will be useful for investigating mechanisms that regulate chief cell physiology in health and disease.

gastric; methods; zymogenic cells

HELICOBACTER PYLORI (HP) or felis infection in animal models including mice, guinea pigs, and Mongolian gerbils has been instrumental in helping to elucidate mechanisms that are important in the pathogenesis of corpus-predominant HP disease, including inflammation, atrophy, metaplasia, and the progression to gastric cancer (9, 11, 14, 23, 32). However, detailed mechanistic studies concerning HP infection in gastric epithelial cells are often not done because of a lack of appropriate culture models. For instance, it is difficult to study how bacterial virulence factors such as vacA and cagA alter cell-specific signaling pathways and function in gastric epithelial cells. In addition, it is currently not possible to study the way in which HP infection alters the gastric mucosal barrier, both at the surface and within gastric glands, or to determine how HP-associated inflammation influences cell-specific survival and death pathways in gastric epithelial cells, especially in chief cells. For such studies, primary epithelial cells in culture that form a confluent monolayer and have intact barrier properties would be required. Primary cells from the gastric mucosa of most species are difficult to isolate and grow in culture and do not form a tight barrier. Because of this limitation, cancer cell lines or transformed cells, which may or may not reflect mechanisms that occur in normal gastric epithelial cells, are currently used.

To study the physiology of chief cells at the base of gastric glands, Ayalon et al. (2) developed a technique to produce cultures using chief cells isolated from the canine gastric mucosa. For this, cells isolated by collagenase digestion were purified by centrifugal elutriation to yield cultures with a high transepithelial resistance (TER) and low permeability that maintained differentiated function as shown by agonist-induced pepsinogen secretion (2, 26). Since the procedure was developed, short-term cultures have been produced from isolated human and rabbit (10), guinea pig (25), pig (12), and rat (20, 30, 31) chief cells. Although isolated chief cells and short-term cultured cells from all species secrete pepsinogen (10, 12, 25, 31), it is not currently possible to produce a confluent monolayer of chief cells with a high TER, low permeability, and high rate of agonist-induced pepsinogen secretion from a species other than dog. Because probes are now available to facilitate studies concerned with gastric physiology and pathophysiology in rodent species, development of cultured chief cells from the rat or mouse stomach would be timely and important.

Thus the aim of the present work was to develop a chief cell culture model using the rat stomach. We chose rat because the stomach is relatively large and the isolation and purification procedures yield a considerable number of enriched isolated chief cells. By using hepatocyte growth factor (HGF), isolated chief cells can be seeded at low density in culture to produce a large number of confluent monolayers with one preparation. The resulting cultures grown with HGF have significantly more chief cells. In addition, it is currently not possible to study the way in which HP infection alters the gastric mucosal barrier, both at the surface and within gastric glands, or to determine how

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MATERIALS AND METHODS

Buffers for cell isolation. Medium A contained (in mM) 0.5 NaH₂PO₄, 1.0 Na₂HPO₄, 20 NaHCO₃, 70 NaCl, 5 KCl, 11 glucose, 50 HEPES, 2 Na₂EDTA, and 20 mg/ml BSA. Medium B contained (in mM) 0.5 NaH₂PO₄, 1.0 Na₂HPO₄, 20 NaHCO₃, 70 NaCl, 5 KCl, 11 glucose, 50 HEPES, 20 mg/ml BSA, 1.0 CaCl₂, and 1.5 MgCl₂. Medium C contained (in mM) 0.5 NaH₂PO₄, 1.0 Na₂HPO₄, 20 NaHCO₃, 70 NaCl, 5 KCl, 11 glucose, 50 HEPES, 1 mg/ml BSA (fraction V), 1.0 CaCl₂, 1.5 MgCl₂, and 0.5 dithiothreitol. Medium D contained (in mM) 0.5 NaH₂PO₄, 1.0 Na₂HPO₄, 20 NaHCO₃, 70 NaCl, 5 KCl, 11 glucose, 50 HEPES, 10 mg/ml BSA, 1.0 CaCl₂, 1.5 MgCl₂, and 0.5 dithiothreitol. Media C and D were sterilized before use. BSA (fraction V and globulin free) and all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell isolation and purification. Animals used for this study were maintained in accordance with the guidelines of the Committee on Animals at the Beth Israel Deaconess Medical Center and all animal experiments were done with IACUC-approved protocols. Isolated chief cells were prepared from the rat stomach (Sprague-Dawley; Taconic, Germantown, NY) by density gradient and centrifugal elution techniques as described previously by others (3–7, 20) and modified by us to obtain highly enriched chief cells from the rat stomach. In brief, nonfasted rats, weighing no more than 200 g, were anesthetized with pentobarbital sodium. The stomach was excised, everted, and tied at both esophagus and pylorus. The everted sac was filled with 2 ml of medium A, containing 2.5 mg/ml of protease E (Sigma-Aldrich, St. Louis, MO) and placed in medium A for 30 min in a shaking water bath. The solution was changed to medium B. Surface and parietal cells were harvested at 30 and 60 min, respectively (fractions 2 and 3) and discarded. Chief cells collected 30 min later (fraction 4) and after three 30-s vortex pulses (fractions 5A, B, and C), were combined and then pelleted by centrifugation at 210 g in a Sorval benchtop centrifuge, resuspended in medium C, and then loaded into a JE 5.0 elutriation rotor (Beckman J6-MC centrifuge) and centrifuged at 14,170 rpm for 10 min and then resuspended in a 1:1 mixture of Ham's F-12 medium and Ham's F-12, supplemented with 10% FBS, 2.9 K/ml penicillin, 100 U/ml streptomycin, and 0.25 µg/ml amphotericin B. Cell culture media and serum were obtained from GIBCO-BRL. B. Cell culture media and serum were obtained from GIBCO-BRL.

Chief cell culture. Enriched chief cells were plated at density of 2.9 × 10⁵ cells/cm² in collagen-coated Transwell plates or in collagen-coated culture dishes and incubated in 5% CO₂ in air at 37°C under the following conditions: 1) culture media with no added growth factor; 2) culture media with 100 ng/ml of basic fibroblast growth factor (bFGF); 3) culture media with 100 ng/ml of EGF; 4) culture media with 100 ng/ml of bFGF and 100 ng/ml of EGF; 5) culture media with 100 ng/ml of human gastrin (Mist1), H₂K⁺ ATPase, and HGF; and HGF were purchased from PreproTech (Rocky Hill, NJ).

Immunofluorescence and confocal microscopy. Cells grown on either culture dishes or Transwell filters were fixed for 10 min at room temperature with 4% formaldehyde in 0.1 M Gomori phosphate buffer (pH 7.4), washed with PBS, and then permeabilized with 0.25% Triton X-100 for 4 min at 0°C. Frozen sections of unfixed chief cells were cut and then fixed for 1 min with acetone. Cultured cells or sections were then incubated for 1 h with blocking buffer followed by staining with specific antibodies to 1) pepsinogen II (BioDesign, Saco, ME); 2) occludin (Zymed, South San Francisco, CA) for tight junctions; 3) zonula occludens-1 (ZO-1; direct FITC labeled; Zymed) to outline cell boundaries; 4) gastrin intrinsic factor (GIF, kindly provided by Dr. David Alpers, Washington University, St. Louis, MO), which is a chief cell marker; 5) muc-5AC (Novocastra, Newcastle upon Tyne, UK), which is a differentiated surface cell marker; 6) H⁺-K⁺-ATPase (kindly provided by Dr. Adam Smolka, Medical University of South Carolina), which is a parietal cell marker; 7) vimentin (Novoceastra), which is a fibroblast-specific marker; 8) Ki-67 (DakoCytomation, Carpinteria, CA), which was used to identify proliferating cells; and 9) Mistl (Santa Cruz Biotechnology, Santa Cruz, CA), which is a class B basic helix-loop-helix transcription factor that labels the nucleus of mature chief cells. Alexa 488-labeled Griffonia simplicifolia II (GSI, Molecular Probes) is a lectin probe that was used to quantify mucous neck cells whereas propidium iodide (PI, Molecular Probes) or 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) were used to identify nuclei. All secondary antibodies were purchased from Jackson Immunobiologicals (West Grove, PA).

To determine whether confluent culture cells contained continuous cell boundaries (tight junctions), anti-occludin expression was evaluated by confocal microscopy using a Zeiss LSM510-META confocal system (Carl Zeiss, Thornwood, NY). Cell purity was determined by counting the total cell number, as identified by PI staining of nuclei, against the number of cells stained with specific antibody via a Nikon TE300 microscope (MicroVideo Instruments, Avon, MA) outfitted with an Orca charge-coupled device camera (Hamamatsu Photonics) and IP Lab (Scanalytics, Fairfax, VA) image processing software. Approximately 1,000 cells per slide or dish were evaluated. To determine whether the proliferating cells in culture were chief cells, isolated chief cells were seeded at a density of 2.9 × 10⁵ cells/cm² and incubated for 48 h with 100 ng/ml of HGF. The cells were then fixed, stained for GIF, ZO-1, and Ki-67, and evaluated by confocal microscopy using projected Z-stack images so that all cells coexpressing GIF⁺ granules and Ki-67 in the nucleus could be counted. This experiment was done using three different chief cell preparations. The purity of cultured cells at 48 and 96 h after plating was determined by immunostaining for GIF and ZO-1, and the number of cells containing GIF⁺ granules was determined as a percentage of the total cell number. Images taken by confocal microscopy using projected Z-stack images, as above. This was done using cultured cells from five different chief cell preparations. To determine the composition of chief cell cultures, isolated chief cells were seeded at a density of 2.9 × 10⁵ cells/cm² on Transwell filters and then incubated for 4 days with 100 ng/ml of HGF. The cells were fixed, stained for GSII, GIF, Mistl, and DAPI, and then evaluated by confocal microscopy using projected Z-stack images. This experiment was done by evaluating five different areas from one Transwell filter in three different chief cell preparations; 250 cells were counted per preparation or ~750 cells were evaluated overall.

Electron microscopy. Cultured chief cells grown on plastic chamber slides were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 2 h and then overnight at 4°C. After a brief wash in cacodylate buffer, the cells were postfixed for 1 h in 1% osmium tetroxide containing 1.5% potassium ferrocyanide and then with 2% aqueous uranyl acetate overnight at 4°C. The cells were dehydrated in ethanol and then embedded in LXXI2 resin. Thin sections were collected on Formvar and carbon-coated grids and then examined with a JEOL 1200EX electron microscope. Figures were made from negatives that were scanned to create digital files.

Electrophysiological analysis of chief cell cultures. TER was measured in Transwell filter chambers using the Milli-cell ERS system (Millipore, Billerica, MA). The background resistance of chambers containing medium alone was subtracted from the value of all experimental conditions. TER was evaluated 1–4 days after plating and was measured under all growth factor conditions. Experiments were also done by plating the cells at control density, which is 2.9 × 10⁵ cells/cm² or at high density, which is 13.4 × 10⁵ cells/cm² and
measuring TER at days 1–4 after plating. To determine whether HGF affects the TER of monolayers after they are confluent, confluent monolayers of chief cells were made by plating cells at low density with or without HGF or at high density without HGF. HGF was removed from monolayers for 24 h and then 100 ng/ml of HGF was added to each monolayer, irrespective of whether it had been previously incubated with HGF and irrespective of the initial plating density. TER was measured after incubation with HGF for 24 h.

Measurement of permeability in confluent chief cell cultures. Mucosal (luminal or top well) to serosal (nutrient or bottom well) fluxes of mannitol were done using Transwell filters containing confluent monolayers at day 4. For these studies, 3 mM mannitol was added to the luminal solution and 3 mM D-glucose to the serosal solution. After equilibration for 30 min, 2 µCi of [3H]mannitol (15–30 Ci/mmol, NEN Life Science Products, Boston, MA) was added to the mucosal solution and the cells were returned to 37°C in the incubator. Triplicate wells were sampled for each treatment at 1 and 2 h after the addition of labeled mannitol. The concentration of mannitol in the serosal solution was determined by liquid scintillation as described previously (19). In brief, chief cells in culture were grown to confluence (4 days after seeding) in control conditions or with 0.1–100 ng/ml of bFGF, EGF, or HGF. Additionally, in a culture dish or on a Transwell filter was evaluated with no growth in the culture dish as an estimate of cell number. The number of cells was determined by use of a microplate reader.

Measurement of pepsinogen secretion from isolated chief cells in culture. Pepsinogen secretion was measured by the conversion of pepsinogen to pepsin using hemoglobin as a substrate as was described by Hersey et al. (13). In brief, isolated chief cell cultures were grown to confluence (4 days after seeding) in control conditions or with 100 ng/ml of bFGF, EGF, or HGF and then the growth factor was withdrawn overnight prior to measuring pepsinogen secretion. In pilot experiments, confluent chief cell cultures were able to maintain the same high TER for 2 days (48 h) after withdrawal of HGF. The luminal and serosal media were changed and then the luminal solution was collected at 1 and 2 h to measure basal pepsinogen secretion. For control, bFGF, and EGF, cultured chief cells were stimulated with 1 mM carbachol, which was added to the serosal solution. For HGF, cultured chief cells were stimulated with 1 mM carbachol with or without 1 µM atrazine, a muscarinic acetylcholine receptor antagonist that blocks the action of carbachol. All agonists and antagonists were also added to the serosal solution. At 60 min after stimulation, the entire apical solution (0.2 ml) was extracted and replaced with fresh buffer. Medium with secreted pepsinogen was placed in a Microcon centrifugal filter device (Millipore) and concentrated by centrifugation in an Eppendorf centrifuge. This concentrated solution was then mixed with hemoglobin solution and incubated for 50 min at 37°C. TCA was added to stop the reaction. After spinning to pellet proteins, the resulting supernatant was incubated with cupric ion reagent (0.5% CuSO4, 1% NaK, 2% Na2CO3 in 0.1 N NaOH) followed by Folin reagent (Sigma Chemical, St. Louis, MO). The absorbance was measured at 750 nm by use of a microplate reader and the concentration of pepsinogen in each sample was calculated from a standard curve.

Quantification of cell number. The number of chief cells in culture was evaluated by a colorimetric assay using crystal violet by a method outlined previously by us (19). In brief, chief cells in culture were washed with PBS to remove dead cells, fixed with methanol, air dried, and stained with crystal violet. Stained cells were solubilized and the absorbance was measured at 590 nm by use of a microplate reader. Absorbance values were determined at 590 nm by use of a microplate reader. Absorbance values were 0.001 and denoted by asterisks on the figures.

Statistical analysis. Combined data were expressed as means ± SE. Statistical analysis was done with SigmaStat software (Jandel Scientific Software, San Rafael, CA). The unpaired t-test was used for analysis of two groups; differences in means were regarded as statistically significant at P < 0.001 and denoted by asterisks on the figures. One-way analysis of variance for many groups was followed by multiple comparisons of means when the test for normality passed. If it did not, the Kruskal-Wallis one-way analysis of variance on rank was performed. For both tests, differences in means were regarded as statistically significant at P < 0.05 and denoted as asterisks on the figures.

RESULTS

Rat chief cells require HGF to produce a confluent monolayer with continuous tight junctions. After isolation, the enriched chief cell fraction contained 88.1 ± 1.3% pepsinogen-containing chief cells, of which 9.2 ± 1.4% were GSII-positive mucous neck cells (not shown). Contaminating cells were surface cells (9.7 ± 0.9%), parietal cells (0.23 ± 0.15%), connective tissue cells including fibroblasts (0.33 ± 0.18%), and other cells (1.64 ± 1.57%). When the isolated and enriched chief cell fraction was plated at a density of 2.9 × 10^5 cells/cm^2 in tissue culture media containing fetal bovine serum and hydrocortisone (control condition), cells attached to the bottom of collagen-coated (plastic) dishes by 24 h after seeding and by 2 days showed clusters of attached cells (Fig. 1A). By 4 days after seeding, the dish bottom was covered by a monolayer of cells (Fig. 1B). The cultured chief cells in control conditions were large and flat, with 157.9 ± 8.6 cells/cm^2, and had discontinuous cell boundaries (tight junctions) between many cells (Fig. 2A). Similar results were obtained when the enriched chief cell fraction was grown in culture medium containing serum and hydrocortisone and supplemented with bFGF (Fig. 1, C and D).

In contrast, the addition of EGF or HGF to the enriched chief cell fraction facilitated rapid monolayer formation (Fig. 1, E–H). In the presence of HGF, chief cells attached to the dish and were nearly confluent by 2 days after seeding (Fig. 1G). By 3–4 days after seeding in the presence of HGF, chief cells

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formed a confluent monolayer (Fig. 1H) that contained $309.4 \pm 17.6$ cells/cm², or twofold more cells than in control conditions or with bFGF. With HGF the cells were small (Fig. 2B) and polarized with secretory granules and microvilli at their apical surface (Fig. 3A). Tight junctions were continuous between cells (Fig. 2B) and were located at the apical cell surface (Fig. 3B). Lateral cell membranes were highly interdigitated and also contained desmosomes (Fig. 3B). Unlike chief cell morphology in tissues, however, cultured chief cells were low cuboidal in shape and had small granules, an extensive luminal surface, disorganized apical cytoplasm, and few stacks of lamellar rough endoplasmic reticulum at the basal

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**Fig. 1.** Phase-contrast images of chief cells in culture incubated with no growth factor (A and B) or with 100 ng/ml of basic fibroblast growth factor (bFGF; C and D), EGF (E and F), or hepatocyte growth factor (HGF; G and H) at 2 days (A, C, E, G) or 4 days (B, D, F, H) after seeding. When isolated chief cells were plated at density of $2.9 \times 10^5$ cells/cm², cells attached to the culture dish and by 2 days after seeding had covered the plate with islands of cells separated by bare areas (arrows). With HGF, the monolayer had only a few bare areas at 2 days after seeding (arrow) and was thus nearly confluent. By 4 days after seeding, all chief cell cultures were confluent. Bar (A–H) = 400 μm.
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With no growth factor or with bFGF, chief cell cultures
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166.25
evident as early as 24 h (1 day), when cell number was
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stimulated pepsinogen secretion, which was 82
HGF, from 30–100 ng/ml, had the same effect on TER in
A
25 ng cm
2
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25 ng cm
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25 ng cm
2
HGF increases cell number by stimulating the proliferation
of chief cells to facilitate monolayer formation. When chief cell cultures
were incubated with 100 ng/ml of HGF, the number of
attached cells increased over time to ~200% of control by 4
days after seeding (Fig. 6). This increase in cell number was
evident as early as 24 h (1 day), when cell number was
166.25 ± 24.78% of that found in time-matched control
cultures (not shown). Hydroxyurea, a compound that blocks
cell proliferation by inhibiting cell division, significantly re-
duced the number of cultured chief cells in a concentration-
dependent manner (Fig. 6). These results were obtained by
using the crystal violet assay to analyze cell number and were
confirmed by using tritiated thymidine uptake in the presence
of hydroxyurea, which resulted in uptake values of 63.0, 48.0,
or 30.7% of the value obtained with HGF alone for 1, 3, or 10
mM hydroxyurea, respectively. Hydroxyurea, used at 10 mM
or below, did not affect the viability (95.2 ± 2.7%) of chief
surface (Fig. 3). Chief cell cultures that were grown on Trans-
well filters coated with collagen and incubated with HGF
showed similar characteristics to cells grown on plastic dishes.
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A

Fig. 2. Cell attachments at tight junctions in
chief cell cultures incubated without (A) or
with (B) HGF. Green label is anti-occludin
staining for tight junctions and red is pro-
pidium iodide staining for DNA in the
nucleus. Chief cells were grown to confluence
(4 days after seeding) on Transwell filters in
the absence or presence of 100 ng/ml of
HGF. Cells were fixed directly on the filter
and evaluated by confocal microscopy. Note
that whereas the localization of occludin was
discontinuous in control cultures (arrows),
occludin localized to the entire region of
cell-cell contact in chief cell cultures incu-
bated with HGF. Also note that the cells
lateral membrane from adjacent cells. Desmosomes (D) were also present
with 100 ng/ml of HGF for 4 days. The resulting confluent monolayers
were fixed and processed as described in MATERIALS AND METHODS. Note that
whereas the localization of occludin was
discontinuous in control cultures (arrows),
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cells from the rat form a barrier, we measured TER from
1 to day 4 after seeding and the flux of mannitol at day 4 after
seeding in the absence or presence of growth factors (Fig. 4).
With no growth factor or with bFGF, chief cell cultures
obtained a TER of no greater than 300 Ω·cm² irrespective of
the number of days in culture (Fig. 4A). With EGF, TER
developed on days 3-4 to a maximum of 700 Ω·cm² (Fig. 4A).
The addition of HGF to chief cell cultures resulted in the
highest TER, which by day 3 was nearly 2,000 Ω·cm² (Fig. 4A).
HGF, from 30–100 ng/ml, had the same effect on TER in
chief cell cultures (not shown). Permeability was inversely
correlated to TER (Fig. 4B), where cells incubated with HGF
had 50-fold less permeability than in control conditions.

Chief cell cultures prepared with HGF show a high rate of
stimulated pepsinogen secretion. In chief cell cultures prepared
with HGF, carbachol-stimulated pepsinogen secretion was sig-
ificantly higher than in control cultures or in cultures prepared
with bFGF or EGF (Fig. 5). Under basal conditions with no
stimulation, pepsinogen secretion in all cultures was ~15
ng·cm⁻²·h⁻¹. After stimulation in control cultures or in cultures
incubated with bFGF or EGF, there was a mean rise in
pepsinogen secretion, to ~25 ng cm⁻²·h⁻¹. In contrast, cul-
tures incubated with HGF showed a high rate of carbachol-
stimulated pepsinogen secretion, which was 82 ± 2.5
ng·cm⁻²·h⁻¹. To verify that pepsinogen secretion was spe-
cific, atropine was used to block muscarinic receptors that are
activated by carbachol. With atropine, the rate of carbachol-
stimulated pepsinogen secretion was reduced significantly to
25 ± 1.8 ng·cm⁻²·h⁻¹ (Fig. 5).

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sured as the flux from the mucosal to serosal chambers (JMS).

Chief cells (Fig. 7), anti-ZO-1 was used to outline cell borders
numerous proliferating cells. Anti-GIF was used to identify 48-h chief cell cultures that were not confluent and thus had proliferating cells were chief cells (Fig. 7). This was done in markers for chief cells and proliferating cells to verify that the icated and unable to proliferate, we evaluated the localization of
attachment of cells to the culture dish (not shown).

Because chief cells are thought to be terminally differenti-
ated and unable to proliferate, we evaluated the localization of
markers for chief cells and proliferating cells to verify that the proliferating cells were chief cells (Fig. 7). This was done in 48-h chief cell cultures that were not confluent and thus had numerous proliferating cells. Anti-GIF was used to identify chief cells (Fig. 7A), anti-ZO-1 was used to outline cell borders (Fig. 7B), and anti-Ki67 was used to identify the nucleus of proliferating cells (Fig. 7C). Merged images clearly show that the nucleus of proliferating cells is associated with cells containing robust GIF expression (Fig. 7D).

Cell number influences TER in chief cell cultures. To determine whether there is a relationship between cell number and TER in the chief cell cultures and whether HGF is necessary for monolayer development if high cell numbers can be obtained in other ways, isolated chief cells were plated at J) 2.9 × 10^5 cells/cm^2 without HGF (low density); 2) 13.4 × 10^5 cells/cm^2 without HGF (high density); or J) 2.9 × 10^5 cells/cm^2 (low density) with 100 ng/ml of HGF (Fig. 8A). Interestingly, cells plated at high density without HGF resulted in cultures with significantly more cells in the confluent mono-

Fig. 4. Effect of bFGF, EGF, and HGF on transepithelial resistance (TER; A) and permeability (B) of chief cells in culture. A: isolated chief cells were grown on Transwell filters in media (Control) or in media containing 100 ng/ml of bFGF, EGF, or HGF. TER was measured from 1 to 4 days after seeding, and the data are means ± SE of 3 wells from 8 different experiments. *Significant difference in TER compared with control (P < 0.001). †Significant difference in TER of cells incubated with HGF compared EGF (P < 0.001). B: isolated chief cells were grown to confluence (4 days after seeding) on Transwell filters in control media without growth factors (−GF) or in media containing 100 ng/ml of bFGF, EGF, or HGF. The permeability of ^[3H]mannitol was measured as the flux from the mucosal to serosal chambers (J_mane) each hour for 2 h. Data are means ± SE of 3 wells from 3 different experiments. *Significant differences in permeability compared with control (P < 0.001). Note that chief cell cultures have a high TER and low permeability when incubated with HGF compared with cultures incubated with media alone or with other growth factors.

Fig. 5. Effect of growth factor on pepsinogen secretion in response to carba-chol in chief cell cultures. Isolated chief cells were grown on Transwell filters and incubated with no growth factor or with 100 ng/ml of bFGF, EGF, or HGF. After cells formed a confluent monolayer with a TER of at least 1,800 Ω·cm^2, they were washed in medium without HGF and then incubated in the same medium (without HGF) overnight to prevent any effect the growth factors might have on stimulated pepsinogen secretion. For measurement of basal secretion, the medium was changed and then collected 60 min later. For stimulated secretion, cultures were incubated for 60 min with carbachol (1 mM) that was added to the serosal chamber. For cultures that were grown with HGF, atropine (1 µM) was also coapplied with carbachol to inhibit carbachol-stimulated secretion. Data represent means ± SE from 3 different experiments. There were no significant differences in the basal pepsinogen secretion among groups. *Significant difference in net pepsinogen secretion in response to carbachol compared with the basal condition (P < 0.001). †Significant inhibition by atropine compared with net pepsinogen secretion in response to carbachol (P < 0.001). Note that pepsinogen secretion in response to carbachol was significantly enhanced in chief cell cultures that were grown initially with HGF, and this enhanced secretion was significantly attenuated by atropine.

Fig. 6. Effects of HGF on the proliferation of cultured cells in the presence of hydroxyurea. Isolated chief cells were plated on plastic culture dishes and incubated for 36 h with media alone (Control) or with 100 ng/ml HGF in the presence or absence of 1, 3, or 10 mM hydroxyurea to block cell proliferation. The data are means ± SE from 4 wells from 3 different experiments and are expressed as the percentage of cells that were incubated with no growth factor. Because the data were analyzed by 1-way analysis of variance followed by multiple comparison of means, the significance was tested by our statistics program only at the P < 0.05 level. *Significant increase in cell number compared with the number of cells in control cultures. †Significant decrease in cell number with HGF in the presence of hydroxyurea compared with HGF alone.
layer, and this number of cells was identical to that obtained in cultures prepared by low-density seeding with added HGF (Fig. 8A). Likewise, cultures plated at high density with no added HGF had a maximal TER nearly identical to cultures seeded at low density with added HGF (Fig. 8A). It should also be noted that irrespective of the initial plating density or use of HGF to develop the monolayer, HGF had no effect on TER (Fig. 8B) or the morphology (not shown) of cultured chief cells when it was added to a confluent monolayer.

HGF, when added to cells seeded at low density, produces primary cultures consisting of intrinsic-factor-expressing chief cells that are a mixture of developmental stages. By 2 days (48 h) and 4 days (96 h) after seeding at low density with HGF, chief cell cultures contained nearly all (99.06 ± 1.27%) GIF-expressing cells (Fig. 9, A–D). The expression of GIF was low in some cells and high in others (Fig. 9A), but when ZO-1 was used to outline cell borders (Fig. 9B) it was clear that all cells in the culture contain a few to many granules that stain positive for GIF (Fig. 9C). To evaluate the developmental stage of chief cells that constitute chief cell cultures, we stained with GSII lectin and with anti-GIF and anti-Mist1 to determine the percentage of cells that coexpress these markers (Fig. 10, A–E and Table 1). About 36% of GIF positive cells were negative for GSII and positive for Mist1 whereas 31% of cells were positive for GIF and negative for the other markers (Fig. 10, A–E and Table 1). About 9% of cells were positive for all three markers, and 23% of cells were positive for GSII and GIF but negative for Mist1 (Fig. 10, A–E and Table 1). To determine whether Mist1 was cytoplasmic or had some expression in the nucleus, we imaged nuclei in single-plane confocal sections (Fig. 10F), rather than in projected Z stacks (Fig. 10, C and E). Although a small amount of Mist1 localized to the nucleus, most was cytoplasmic in cultured chief cells. This result was in contradistinction to fixed and stained rat tissues, which we did originally to test the antibody, where Mist1 expression was nuclear in all chief cells (not shown).

**DISCUSSION**

In the present study, we describe a technique for making highly purified chief cell cultures from the rat stomach that have a high TER, low permeability, and a high rate of stimulated pepsinogen secretion. Although this has been accomplished before with chief cells isolated from the dog stomach (2, 26, 27), such a culture system in rodent species has not been described. In previous studies using chief cells isolated from the rat stomach, cultures were made either by using mixed mucosal cells with no purification (30, 31) or by preparing highly purified chief cells (85 ± 11% pure) by elutriation and Percoll density gradient centrifugation (20). Chief cell cultures made from these highly purified cells were used 2 days after seeding, but it was not mentioned whether they were confluent.
with the “low density” seeding we did for rat chief cells, which were plated at 2.9 × 10^5 cells/cm^2 (4.6-fold fewer cells). Because the rat stomach is small and few highly purified cells are isolated from eight rats, only nine wells of a 12-well Transwell plate can be plated at high density and thus few experiments can be done with one preparation. In contrast, low-density seeding allows for the preparation of four 12-well Transwell plates from each preparation. Using HGF to increase the number of chief cells by about twofold was postulated by us to be one important feature of HGF that allowed for the successful production of rat chief cell cultures with the new protocol. To support this notion, we showed that purified rat chief cells plated at a high density of 13.4 × 10^5 cells/cm^2 in the absence of HGF formed a confluent monolayer that had the same number of cells and TER as occurred when cells are plated at low density with HGF. This result suggests that HGF mediates an increase in cell number that is, in some way, essential to develop chief cell cultures and that HGF, per se, is not required for chief cell propagation in vitro. Although it was postulated that HGF would protect chief cells from dying after isolation, as occurs in gastric RGM1 cells by induction of the cyclooxygenase-2 (COX-2) gene and prostaglandin E2 (PGE2) synthesis (29), HGF did not serve this function in chief cells (not shown). Our results also demonstrate that HGF is required only for the development of chief cell cultures; it can be withdrawn and the TER remains high for at least 2 days (48 h) postconfluence. We also showed that chief cell cultures plated initially in control conditions will not develop a high TER if HGF is added to cells after they are confluent, supporting the contention that HGF is necessary only for developing chief cell cultures. At present, it is not known how an increase in cell number improves pepsinogen secretion or barrier function of chief cells in culture. Because it is necessary to seed a high density of cells or to seed fewer cells but provide HGF, the strategy used for preparing cultured chief cells may depend on how many cells can be obtained in the initial isolation procedure.

One surprising outcome of the present study was that cultured chief cells express markers for mature, immature, and transitional cells as described by Ramsey et al. (24) and as such are a mixture of different developmental subtypes. We found, however, that a number of developmental stages in our cultured cells were either over- or underrepresented compared with the intact gland, in vivo. For instance, Ramsey et al. showed that 97% of mature chief cells have the marker profile GSII^-GIF^+Mist1^+ whereas 3% were Mist1^- . The latter population is expressed at levels 10-fold higher in our cultures (>30% GSII^-GIF^-Mist1^-); the reason for this result is unknown. Furthermore, we show that only 9% of cells in chief cell cultures express the marker profile GSII^-GIF^-Mist1^+, which in vivo is a marker profile that represents immature chief cells. This result was unexpected considering that when parietal cells are deleted from the gastric corpus in vivo, recapitulating the condition found in highly pure cultured chief cells in vitro, the marker profile of gland cells changes to TFF2^+ (a neck cell marker like GSII) GIF^+Mist1^+. SPEM cells (21). It is possible that more than 9% of cultured chief cells are SPEM cells, however. Mist1 was recently shown to be downregulated over time in the absence of parietal cells (21) and thus the 23% of cultured chief cells that express the

or not. Additionally, it was demonstrated that cultures made from highly purified chief cells showed a significant reduction in the expression of some proteins found in mature chief cells in vivo (20). Our results show that chief cells in culture must be confluent with a high cell density to, both functionally and structurally, express a phenotype that is similar to chief cells, in vivo.

Initially, it was hypothesized by us that difficulties in growing chief cell cultures from rat, rather than from canine species, may be due to the minimal number of chief cells that are isolated in each preparation and available for plating in culture. For instance, chief cell cultures from the dog stomach were seeded, by our calculation from the original paper (2), at a density of 13.4 × 10^5 cells/cm^2 (“high density”) compared
marker profile GSII+GIF+Mist1− may represent either transitional cells or a population of Mist1−SPM cells. Mist1 was recently shown to be essential for development of mature chief cells, which are pyramidal in shape, have large secretory granules and an elaborate supranuclear cytoplasm, and develop a well-organized system of rough endoplasmic reticulum (rER) (24). Despite the fact that nearly 50% of cultured chief cell in our study express Mist1, they do not show this mature morphology. Instead, cultured chief cells are low cuboidal cells with small granules and have a disorganized cytoplasm without a highly organized rER. This also appears to be the morphology of canine chief cells in culture (2). The cytoplasmic localization of Mist1 that we show here inhibits its function as a nuclear transcription factor, most likely resulting in chief cells that are unable to develop into fully mature cells. The lack of nuclear Mist1 localization may also explain why chief cells, which are thought to be terminally differentiated cells that do not divide, proliferate vigorously in the presence of HGF. This is because nuclear Mist1 expression was recently shown to block and downregulate p21(CIP1/WAF1) cyclin-dependent protein kinase activity and proliferation in pancreatic acinar cells (15). If Mist1 has a similar function in chief cells, the proliferation blockade by Mist1 in mature chief cells would be lifted as the isolated cells redistribute Mist 1 to the cytoplasm where it cannot transcriptionally downregulate important proliferation markers. Although little is known about how proliferation is regulated in chief cells, it may well be that whatever facilitates the expression and nuclear translocation of Mist1 in chief cells also regulates the proliferative state of these cells. Further work will be required to investigate this interesting idea.

In light of the known effects of HGF as a potent scatter factor for gastrointestinal epithelial cells, in vitro (22), we were puzzled by the fact that HGF did not act to scatter chief cells in culture. In most epithelial and endothelial cells, including MDCK, T84, HUVEC, and the cancer cell lines MDA, MB 231, and MCF-7, HGF disrupted tight junctions in confluent cultured cells to facilitate disassociation of cells and a migratory phenotype (16–18, 22). In HUVEC cells, the effects of HGF on tight junction integrity also resulted in an increase in paracellular permeability (17). Disassociation of gastric cancer cells (AGS cells) also occurred with HGF or when the CagA protein from HP activated c-Met receptor signaling, causing AGS cells to disassociate and form elongated cells with a “hummingbird” phenotype (8, 28). The work presented here clearly shows that HGF added to confluent chief cells in culture does not cause cells to disassociate nor does it affect barrier properties of the monolayer. These results suggest that the role of CagA/c-Met in regulating epithelial transformation during HP infection may not pertain to chief cells.

In conclusion, we demonstrate a new technique to grow isolated chief cells in culture from the rat stomach that have a
high TER, continuous tight junctions, low permeability, and stimulated pepsinogen secretion. The cultures are also easy to produce. Success of the procedure relies on using HGF, which is thought to be a potent scatter factor but facilitates monolayer development in chief cells by facilitating their proliferation. By using the technique outlined here, it will be possible in the future to study, in a reductionist manner, chief cell physiology in isolated and cultured cells from the rat where numerous reagents and probes are available and tested to be species compatible.

Table 1. Components of chief (zymogenic) cell cultures

<table>
<thead>
<tr>
<th>Marker</th>
<th>Marker Is Present or Absent</th>
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<tbody>
<tr>
<td>GSII</td>
<td>–</td>
</tr>
<tr>
<td>GIF</td>
<td>+</td>
</tr>
<tr>
<td>Mist1</td>
<td>+</td>
</tr>
<tr>
<td>Developmental stage</td>
<td>Mature zymogenic cells</td>
</tr>
<tr>
<td>Percentage</td>
<td>36.4±4.2%</td>
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</tbody>
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+, Present; –, absent; GSII, *Griffonia simplicifolia* II to identify gastric neck cells; GIF, gastric intrinsic factor to identify chief cells; Mist1, a class B basic helix–loop–helix transcription factor; TZ, transition zone. Percentage is the number of cells within cultures that express the different combination of markers.
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