Simple and efficient method for isolation and cultivation of endoscopically obtained human colonocytes

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Seidelin, Jakob B., Thomas Horn, and Ole H. Nielsen. Simple and efficient method for isolation and cultivation of endoscopically obtained human colonocytes. Am J Physiol Gastrointest Liver Physiol 285: G1122–G1128, 2003; 10.1152/ajpgi.00533.2002.—Few comparative and validated reports exist on the isolation and growth of colonoscopically obtained colonic epithelium. The aim of this study was to develop and validate a simple method for the cultivation of colonoscopically obtained colonocytes. Forty patients, who underwent routine colonoscopy and where the diagnosis of irritable bowel syndrome was later reached, were included. Seven colon biopsies were taken and incubated at varying time periods of 10–120 min and temperatures of 4–37°C in a chelating buffer. The epithelium was then harvested and cultivated under three different conditions: 1) on a collagen coating, 2) embedded in a collagen gel, or 3) embedded in a gel put on a porous well insert. The effect of conditioned medium (CM), insulin, transferrin, selenium, and the oxygen content was assessed. Viability was tested by the metabolic reduction of methylene blue, by flowcytometry, by phase contrast microscopy, and by transmission electron microscopy. Incubation at 21°C for 75 min gave an optimal yield of 3 × 10^6 (2.0–3.8 × 10^6) viable epithelial cells in intact crypts per seven biopsies. Embedding of crypts in a collagen gel put on a porous membrane was superior to the other methods applied [P < 0.003; median viability 71% (62–100%) compared with preculture values] after 24 h, which was a 160% increase in viability compared with coat-cultivated cells. CM had similar viability supporting effects to FCS. Other supplements had no effects.

A simple method is presented, which makes cultivation of colonocytes obtained at endoscopy possible for up to 72 h. This method is applicable to the diagnosis of inflammatory bowel disease or colonic neoplasia.

METHODS

Patients. Forty patients with the diagnosis of irritable bowel syndrome (14) were included. All patients had a routine colonoscopy or sigmoidoscopy performed as a part of their planned examination program. Patients under the age of 18 or pregnant patients were excluded. No patients received treatment with drugs known to influence the functions of the gut investigated.

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chloroquine, 100 μg/ml gentamycin, and 125 μg/ml amphotericin B. The osmolarity was adjusted by adding Hanks’ balanced salt solution, and pH was adjusted to 7.4 with 1 M NaOH, and the final FCS/CM concentration was set to 15%. The mixture was kept at 4°C to avoid premature polymerization. After the addition of the colonocytes, the gel (100 μl) was allowed to polymerize by incubation for 5 min at 37°C, and the growth medium was finally added to adjust the total final volume to 400 μl.

Assessment of cell numbers. Cell numbers were determined by measurement of the DNA content after Hoechst 33258 staining. The cells were lysed and sonicated to liberate the DNA and incubated with the DNA stain, and the fluorescence thereafter was measured (DyNA Quant 200 Fluorometer, Amersham Biosciences Europe, Freiburg, Germany). A standard of known DNA concentration was included to calculate

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<th>Table 1. Chelation time and temperature</th>
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Efficiency of the chelation procedure with varying time and temperature conditions. Crypt release depicts number of released crypts per 7 biopsies. Crypt morphology describes the appearance of the released crypts as seen in the phase-contrast microscopy: intact, crypt structure conserved; poor, only fragments of crypts seen. No crypts seen, only single cells released. ND, not determined. See Results for further description.
Viability assessment by the dimethylthiazol-diphenyl-tetrazolium bromide test. Viability was assessed by the reduction of dimethylthiazol-diphenyl-tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase to the colored formazan during 4 h of incubation, as previously described in detail (9). Viability was assessed after 24 h of culture and in one experiment at 0, 24, 48, 72, and 96 h of culture.

Viability assessment by flowcytometry. Apoptotic nuclei (sub-G0) have less DNA-content than 2n diploid (G0 or G1) or 4n diploid nuclei (G2) of normal cells, and thus stain less intensively with DNA dyes. Viability was detected by measuring the fraction of viable cells in the G0/1 and G2 peak by flowcytometry, as earlier described in detail (13, 21). In brief, the gel was digested by collagenase (200 units/ml; Sigma-Aldrich) to solubilize colon epithelial cells. The cells were then spun down (500 g; 5 min) and incubated at 4°C for 3 h in a nuclear extraction buffer containing the fluorescent DNA stain propidium iodide, 0.1% Triton X-100, 50 μg/ml propidium iodide. The propidium iodide fluorescence of the individual nuclei was determined by flowcytometry (FACScan, BD Biosciences, San Jose, CA). Viability was assessed at 0, 24, and 48 h of culture.

Viability assessment by electronmicroscopy and assessment of cell types. The colonocyte viability and relative numbers of different cells were assessed by transmission electron microscopy (EM). Cells were fixed in 10% buffered formalin, embedded in paraffin and stained with toluidine blue. Subsequently ultrathin sections were mounted on 150 mesh copper grids and stained with uranyl acetate-lead citrate before examination under a Philips transmission EM EM210 (Amsterdam, the Netherlands). Photomicrographs were taken at ×2,000–3,200. More than 50 cells were counted per single experiment and classified by type (e.g., absorptive, goblet, and stem cells).

Determination of purity of isolated cells by immunohistochemistry. The isolated cells were embedded in a gel as described above, and then they were fixed in 10% buffered formalin, embedded in paraffin in a standard manner, and stored until analysis. This was done after 0, 24, and 48 h of culture.

Immunostaining was performed with a panel of cell type-specific markers. These were epithelial markers: anti-cytokeratin 18 (CK18; clone DC10, DakoCytomation; Glostrup, Denmark), anti-CK20 (clone KS20.8; DakoCytomation), and the pan-specific anti-CK antibody (clone AE1/AE3; Immunotech, Paris, France); mesenchymal cell marker: anti-vimentin antibodies (clone Vim3B4; DakoCytomation); and endothelial cell marker: anti-CD34 antibodies (clone My10; BD Biosciences). The antibodies were used in the following concentrations: CK18: 1:400; CK20: 1:400; CK-PAN: 1:300; Vimentin: 1:400; CD34: 1:100. A two-step method was employed using the EnVision+ visualization system (DakoCytomation). In brief, 5-μm-thick sections were cut, deparaffinized, and hydrated in graded ethanol-water washes and pretreated with microwaves and 3% H2O2. The primary antibodies were applied overnight at 4°C following washes. The EnVision+ system was then applied for 30 min. After washes, the substrate dianinobenzidine was applied to yield a colored product. The slides were counterstained with Mayer’s hematoxylin.

Statistics. Nonparametric statistics were applied. In comparing groups from the individual experiments, the Wilcoxon’s signed rank test was used. The Spearman correlation coefficient was calculated for the DNA measurement experiments. Values were shown with 95% confidence intervals or given as medians and ranges, and a significance level of 0.05 (2α) was chosen.

RESULTS

Isolation procedure. The isolated crypts were evaluated on the basis of relative numbers of crypts liberated and the quality of these crypts in terms of crypt structure preservation and absence of apoptotic cells. In general, the shorter and the colder the duration of the chelation, the more intact were the crypts. The yield, however, had an inverse relationship, i.e., the longer and warmer incubation conditions, the more crypts were liberated (Table 1). No change of crypt architecture was, however, revealed when incubation was performed at 21°C for <75 min, whereas the yield dramatically increased at incubation times >60 min. On the other hand, even a 10-min incubation at 37°C gave crypts with prominent apoptotic features (Fig. 1). Incubation at 4°C for 2 h gave very low yields. As a result, an incubation time of 75 min at 21°C was found to be optimal.

Phase-contrast imaging of the cells released after 75 min at 21°C revealed almost only epithelial cells arranged in crypts and only few single cells. Immunohistochemistry of the released cells showed almost all
cells (>99%) to be positive for CK18 and CK20 (Fig. 2). No vimentin or CD34-positive cells were found. EM revealed crypts to contain all cell types normally found in the colon crypts: goblet cells were predominant (45% of all cells) in the middle part of the crypt, whereas secretory cells dominated the mouth and plateau zones of the epithelium. Less-differentiated cells were encountered at the crypt base. No mesenchymal-like cells were found in the EM sections.

**Determination of cell content.** There was a correlation between crypt numbers and the DNA content [see Fig. 3; correlation coefficient 0.85 (0.68–0.93); P < 0.0001]. No interpatient variability was found, and a simple crypt counting was, in this setting, an estimate of the relative cell number. From the DNA measurements, it could be calculated that the yield was 3 × 10^6 (2.0–3.8 × 10^6) epithelial cells per five biopsies at the chosen chelation conditions.

**Culture methods.** Embedding of the epithelial cells in a three-dimensional collagen structure gave a considerably higher viability of the cells than growing them on a collagen coating (40% better viability; P < 0.02). The viability was even higher in cells embedded in a collagen gel and grown on a porous membrane (60% higher viability; P < 0.01; Fig. 4). The median viability of the colonocytes at 24 h of culture was 71% (62–100%; median (range)) of the preculture value. Cells grown in CM had viability comparable with those grown in FCS-supplemented medium, and no statistical significant difference was found between those two. The median viability was 78% (77–100%) after 24 h (Fig. 5). Neither growth factor supplementation nor increased oxygenation gave a viability benefit (Fig. 6). This was equally found on cells cultured on coated wells, in gels, and in gel plus membrane insert (data not shown). Viability was stable within the first 72 h and decreased only substantially from 72 to 96 h. Thus viability was 58% (46–100%) at 48 h, 55% (40–60%) at 72 h, and 25% (22–36%) at 96 h (Fig. 5). Flowcytometry of propidium iodide-stained nuclei confirmed the results found by MTT (Fig. 7).

**Immunohistochemistry at 24 and 48 h** showed that >99% of cells were CK18 or CK20 positive. No cells of mesenchymal origin were found. EM confirmed no major change in the relative number of different cell types.
Viability confirmed by transmission EM. No significant morphological changes were found in colonocytes cultured for 24 h, compared with those freshly isolated. Cultured cells were all epithelial and revealed normal morphology (Fig. 8). Cells were detached from the basal lamina, and cultures did not contain mesenchymal cells. However, as expected, random sections revealed more apoptotic cells in the cultivated specimens than in the noncultivated.

DISCUSSION

This study provides a new efficient method for the short-term monoculture of colonoscopically obtained colonic epithelial cells organized in preserved crypt structures. The method is optimized in terms of epithelial cell-isolation technique, culture technique, and culture medium additives. The method is compared with previously published methods.

The aim of the isolation procedure is to obtain a pure fraction of viable colonocytes. Any potential apoptosis-inducing condition should therefore be minimized. Isolation has been performed by enzymatic digestion of the connective tissue (4) and by chelation, which disrupts Ca\(^{2+}\)-dependent cell-matrix interactions (24). Chelation has previously been shown to be superior to enzymatic digestion with regards to the purity of epithelial cells and has additionally been shown to cause less apoptosis (10). It is shown in the present study that chelation time and temperature must be optimized to yield crypts without apoptosis features. The optimization is restrained by the inverse relationship between these two variables as shown in Table 1. However, the combination of a long incubation time (i.e., 75 min) and a low incubating temperature (21°C) gives both a high yield and a high viability. As detachment from the basement membrane induces apoptosis (20), the time and temperature should be kept low when cells are in suspension. Even when the cells are embedded in the unpolymerized collagen gel, mechanical stresses such as vigorous mixing should be avoided, because crypt tears will result in decreased viability due to apoptosis (data not shown). The gentle handling of the crypts can, on the other hand, result in some variation in the cell number of each well. It is here shown that simply counting crypts gives a good estimate of cell numbers compared with DNA content measurements. The cells obtained by this method are a highly pure fraction of colonic epithelial cells with very low contamination of mesenchymal cells.

From the present study, it is clear that the gel embedding is substantially superior to collagen coating alone. Culture with crypts embedded in a collagen gel and on porous membrane well insert is further advantageous to culture on the bottom of the well. A viability of 46–100\% within the first 48 h was achieved with this new method, which is substantially higher than other culture methods (16). This may be due to a three-dimensional epithelium-matrix interaction and an improved exchange of metabolites on all sides of the crypts, because crypts will fall to the bottom of the well before the gel has polymerized. The collagens used in this study contain mainly collagen type I of nonhuman origin. Other more basement membranelike substances, e.g., Matrigel, which contains collagen type IV, have previously been tried without beneficial effects on the cultivation process (19). Accordingly, the collagen I/\(\beta1\)-integrin interactions seems to provide the required survival signaling in the colonocyte and inhibit detachment-induced apoptosis; such an effect has not been shown for collagen type IV (20). Medium was not changed in this experiment during the cultivation period. Medium shifting may, however, improve viability during long-term cultivation, i.e., >96 h.

Heterologous-conditioned medium is here shown to be at least as effective as FCS in supporting viability of
isolated colonic cells grown in three-dimensional gels. The median viability was higher in the CM-treated cells, but this difference failed to reach statistical significance. Cells grown without either FCS or CM have a poor viability. These ill-defined additions seem to contain growth factor(s) or other mediators that enhance viability. It has recently been shown that Winslow/INT-1 signaling may be pivotal for stem cell survival in the intestinal epithelium (2, 23). The source might be myoepithelial cells close to the epithelial cells, thus emphasizing that enterocyte monoculture requires both extracellular matrix proteins and compartment-specific mediators. The effect of CM was, however, not as profound as that found by Panja (15). The intestinal epithelial cells used in this study were, however, very different from those isolated in this study, mainly by being passed several times after isolation. They may most likely represent a stem cell-like cell type with a high dividing potential and could thus be more responsive to CM-derived mitogens. The crypt cells used in this study are mainly differentiated cells without a dividing potential, and stem cells may account for <5%, which could explain the weaker effect of CM (17). Addition of EGF, insulin, transferrin, or sodium selenite did not enhance viability in this study. Only a few comparative studies on culture media for colonocytes have been performed (5, 6, 18, 19, 25). Various growth factors have been used; however, the effects have only been poorly investigated. The supplements include transferrin and EGF (5), insulin (4, 5, 19), and/or hydrocortisone (4, 19, 24). EGF has been shown to be beneficial in three studies (5, 6, 19), to impair proliferation in one study (5), and to have no effects in other studies (18, 25). Conflicting results have also been found with insulin supplementation (5, 18, 19). Other growth factors have been shown to have no effects: amphiregulin, β-regulin, hereregulin, pentagastrin, bombesin, hepatocyte growth factor, or insulin-like growth factors 1–3 (19, 25). Lastly, transforming growth factor-β has been shown to be beneficial (6). In the present study, EGF, insulin, transferrin were applied at concentrations similar to the ones mentioned in the above studies.

It is essential to recognize that isolation procedures have profound effects on the viability in subsequent cultures and that variations of these procedures may account for some of the inconsistencies mentioned above, which refer to data from studies that have employed widely differing isolating procedures. Thus many of the growth factors mentioned above are known to inhibit or retard detachment-induced apoptosis (3). This could explain why some of the growth factors enhance viability in some studies, because they could be retarding detachment-induced apoptosis. In addition, some isolation procedures let a substantial fraction of nonepithelial cells pass on with the epithelial cells, most likely fibroblasts and myofibroblasts, which are known to be supported by some of the mentioned growth factors (12).

In conclusion, a new method is provided that permits growth of highly purified and viable human colono-

cytes. This method is superior to others in terms of cellular viability (16). Furthermore, the method allows for the cultivation of freshly isolated morphologically well-preserved colonocytes in a three-dimensional structure that resembles in vivo conditions. The method has made it possible to develop a method by which apoptosis can be observed in living colonocytes over time based on loading of the cells with fluorescent caspase-3 substrates. The preserved crypt structure makes categorizing of dying cells possible, i.e., whether apoptosis occurs in the stem cell region or in differentiated cells. Furthermore, ultrastructural expression analyses with immunogold labeling have been carried out. The method is stable and simple, because it is solely based on routine colonic biopsy samples obtained by endoscopy.

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**DISCLOSURES**

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