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Culturing explanted colon crypts highly improves viability of primary non-transformed human colon epithelial cells

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ABSTRACT

Chemoprotective effects of nutritional compounds are usually studied in cell lines. Studies using primary human colon cells have been limited due to the lack of established methods regarding their culture. We therefore optimized isolation and culture of non-transformed human epithelial cells from individual donors to enrich viable cells and sufficient amounts of intact RNA. Isolated epithelial cells were seeded in different coated cell culture dishes combined with several media (2–24 h). To avoid cells from anoikis, also intact colon crypts were isolated to maintain cell interactions. These crypts were incubated with gut fermentation products (24 h) derived from indigestible carbohydrates. In none of the coated (fibronectin, laminin) cell culture dishes isolated epithelial cells did attach. The number of these cells remaining in suspension, decreased already after 2 h to 20%. Intact colon crypts cultured as pellets showed a stable viability up to 24 h (91 ± 4%) and were suitable to gain a sufficient quantity of RNA. The use of colon crypts with an appropriate cell culture medium could double the lifespan of intestinal epithelial cells from 12 up to 24 h and represents a promising approach to study early events in carcinogenesis and chemoprevention as well as other diseases of the colon.

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1. Introduction

Epithelial cells of the large intestine are permanently exposed to compounds derived from diet and digestive processes including genotoxic carcinogens that are putative risk factors for colorectal cancer (Kaeffer, 2002; Wakabayashi et al., 1992; World Cancer Research Fund and American Institute for Cancer Research, 2007). In the last decade chemoprevention using protective nutritional factors like dietary fiber or polyphenols (Bingham, 2006; Mahmoud et al., 2000) has reached great importance to fight against colorectal cancer (Das et al., 2007). But, especially studies to the underlying mechanisms have been limited due to the lack of suitable cell models of colonic epithelial cells. Many investigations therefore used tumor cell lines such as HT29 and CaCo-2 or adenoma cells such as LT97 (Borowicki et al., 2011; Miene et al., 2011; Rousset, 1986). Those cells are well suited for research of chemopreventive activities in existing pre-neoplastic lesions but less useful for prediction of chemoprotective effects in healthy, non-transformed colon cells (Fonti et al., 1994). Other groups used non-tumorigenic colon epithelial cell lines like NCM460 (human normal colon mucosa) (Moyer et al., 1996) or FHC (human fetal colon) (Hofmanova et al., 2005) cells for their studies. However, they also do not represent a real alternative, since these cells are conditionally immortal (Fenton and Hord, 2006) and have lost several organ-specific functions because of their dedifferentiated phenotype (Hidalgo, 1996). For these reasons the isolation and culture of primary colonocytes is preferred. Many investigators have already developed culture methods for intestinal cells derived from a variety of animal species due to the better availability of such colon tissue. Most of these studies were performed by using tissue of mice (Booth et al., 1995), rats (Evans et al., 1992; Kaeffer, 2002), rabbits (Benya et al., 1991) or bovines (Föllmann et al., 2000). So far, studies which compare the interspecies variation between cultured animal and human colonocytes do not exist. Hence, the comparability of these results is rather problematic. Thus, specific investigations using human primary cells are required. Such studies are able to generate the most relevant results. So far, studies have been limited due to the lack of long-term cultures of colon epithelial cells. The main reason is that *in vivo* half of the mucosa cells are in a terminally differentiated, resting state and already predetermined to die (Garrison et al., 2009). The human colonic

Abbreviations: DAB, diaminobenzidine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; MEM, minimal essential medium; SFS, Synergy1® fermentation supernatant; RIN, RNA integrity number.

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mucosa is characterized by the presence of crypts (14,000 crypts/ cm^2) (Ricci-Vitiani et al., 2009) forming finger-like invaginations into the underlying tissue of the lamina propria (Cheng et al., 1984). Each of them contains from 250 (Booth and Potten, 2000) up to 2000 cells (Nicolas et al., 2007) in total, consisting of a mixture of undifferentiated stem cells, proliferating and differentiated cells. Stem cells are located at the bottom of each crypt. The real number of these cells is still in discussion and data point from probably 1 cell to 20 cells per crypt or approximately 1% of all crypt cells (Potten and Loeffler, 1990). Until now, most investigations on defining location, number and function of intestinal stem cells were performed in murines. The most important step was the discovery of well validated stem cell markers like Lgr5 (Barker et al., 2007). In recent years, especially Clevers and his colleagues carried out intensive research in the field of intestinal stem cells. They estimated the number of stem cells in the small intestine between four and six (Sato et al., 2009). As each stem cell produces a large number of transit and differentiated cells, slight changes in the number of stem cells have important implications for maintenance of the integrity of the crypt. Therefore, it is an important aim to cultivate epithelial cells including stem cells. Intestinal stem cells represent the actual target cells of carcinogenesis since they are defined as long-lived and therefore at risk of incurring the series of somatic mutations leading to carcinoma. All other intestinal epithelial cell types are short-lived (Cammareri et al., 2008). Intestinal stem cells are highly sensitive to anoikis, a special form of apoptosis (Frisch and Francis, 1994). Thereby, it has not been possible to cultivate isolated colon stem cells which could be used as indicator cells for *in vitro* toxicological investigations (Berlau et al., 2004). Until now, primary colon cells in suspension culture remained viable for only short time periods of about 12 h (Sauer et al., 2007). Attachment of epithelial cells to the extracellular matrix plays an important role in the regulation of cell growth and differentiation. Particularly fibroblasts are a source of matrix proteins, especially collagens and fibronectin. The gut basement membrane, a specialized extracellular matrix, is produced by epithelial cells as well as mesenchymal cells in the intestine (Basson, 2003; Kalabis et al., 2003). Loss of cell–cell contact and detachment induces anoikis (Frisch and Francis, 1994; Strater et al., 1996). Therefore, an adequate *in vitro* microenvironment is necessary for optimal culture of colonic epithelial cells. The aim of the present study was to investigate different culture conditions to prolong the colon epithelial cells' life, including that of stem cells for at least 24 h to enable further examinations of potential harmful and protective compounds for incubation periods longer than the current 12 h with adequate cell viability and sufficient amounts of intact RNA for gene expression analyses. Thus, we analyzed relevant factors such as various cell isolation procedures and culture conditions that might influence cell attachment and survival of primary colon cells.

2. Materials and methods

2.1. Primary colon tissue preparation

Normal human colonic mucosa was obtained from 24 patients undergoing surgery of colorectal tumors or other gastrointestinal diseases like colon polyps and diverticulitis. Mean age of the donors was 67.5 ± 9.2 years; 13 of the donors were male and 11 were female. The study was approved by the ethic committee (approval no. 1601-08/05) of the Friedrich-Schiller-University Jena and all patients gave their informed consent.

Normal colon tissue (about 3–7 cm^2), which was macroscopically and microscopically determined as non-malignant, was taken at least 10 cm away from the side of tumor or inflammation. The colon tissue samples were directly stored in Hank's balanced salt solution (HBSS; 8.0 g/l NaCl; 0.4 g/l KCl; 0.06 g/l $Na_2HPO_4 \cdot 2H_2O$; 0.06 g/l K_2HPO_4; 1 g/l glucose; 0.35 g/l $NaHCO_3$; 4.8 g/l Hepes; pH 7.2) after removal and transported on ice from the hospital to the laboratory within 1 h. After extensive washing in HBSS epithelial strips (0.3–0.5 cm^2) were stripped from the submucosa by perfusion supported mechanical disaggregation as described previously (Schaeferhenrich et al., 2003) and rinsed with HBSS several times. The resulting epithelial strips were used for isolation of single cells and colon crypts.

2.2. Isolation of single cells

Single cells were isolated from epithelial strips by mincing followed by enzymatic digestion with 6 mg proteinase K (Sigma, Steinheim, Germany) and 6 mg collagenase P (Boehringer, Mannheim, Germany) dissolved in 5 ml HBSS (90 min, 37 °C). Undigested tissue pieces were removed by passing the solution over a common household sieve. The obtained suspension containing single cells was washed with HBSS, centrifuged (5 min, 4 °C, 100*g*) and resuspended in HBSS. Number and viability of the cells were determined by trypan blue exclusion test.

2.3. Cultivation of single cells in laminin- and fibronectin-coated cell culture dishes

A low attachment rate is a strong limitation factor for culturing of intestinal cells. Laminin and fibronectin are extracellular matrix proteins which should enhance the attachment of the cells (Mahida et al., 1997). Commercially available laminin- and fibronectin-coated cell culture dishes (60 × 15 mm, Greiner bio-one, Frickenhausen, Germany) were used to improve the attachment of the intestinal cells in culture. After centrifugation of single cells, the pellet was resuspended in a primary cell culture medium according to Rogler. This medium consisted of minimal essential medium (MEM) with Earle's salts enriched with 20% fetal calf serum (FCS), 2 mM glutamine, 100 μg/ml gentamycin, 2.5 μg/ml fungizone, 10 ng/ml epidermal growth factor (EGF), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Rogler et al., 1998). The suspension of epithelial cells was seeded at a density of 2×10^6 cells in uncoated, laminin- and fibronectin-coated cell culture dishes and was cultivated for 24 h (37 °C, 5% CO_2, 95% humidity).

2.4. Suitability of several cell culture media

Single epithelial cells were isolated as described before. The cell suspension was seeded in 6-well plates (culture area 9.6 cm^2/well, nunc, Roskilde, Denmark) at a density of 2×10^6 epithelial cells per well. We compared viability, number and attachment of cells cultured in medium according to Rogler and MCDB (molecular, cellular and developmental biology) 302 medium (Biochrom, Berlin, Germany) containing 20% L15 Leibovitz medium, 2% FCS, 0.2 nM triiodo-L-thyronine, 1 mg/ml hydrocortisone supplemented with 10 mg/ml insulin, 2 mg/ml transferrin, 5 nM sodium selenite, 30 ng/ml EGF and 50 mg/ml gentamycin. M3:10 culture medium (Incell, San Antonio, Texas) was used as a third medium.

Viability and cell number were determined after 2, 12 and 24 h by trypan blue exclusion test. Attachment was assessed by microscopical estimation of the cell confluence using an Axiovert 25 (Carl Zeiss AG, Jena, Germany).

2.5. Suitability of laminin-coated cell culture dishes in combination with several cell culture media

In a further series of experiments we tested the addition of several media to laminin-coated cell culture dishes. Single epithelial

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