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Human colon tissue in organ culture: preservation of normal and neoplastic characteristics

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Abstract

Normal and neoplastic human colon tissue obtained at surgery was used to establish conditions for organ culture. Optimal conditions included an atmosphere of 5% CO₂ and 95% O₂; tissue partially submerged with mucosa at the gas interface; and serum-free medium with 1.5 mM Ca²⁺ and a number of growth supplements. Histological, histochemical, and immunohistochemical features that distinguish normal and neoplastic tissue were preserved over a 2-d period. With normal tissue, this included the presence of elongated crypts with small, densely packed cells at the crypt base and mucin-containing goblet cells in the upper portion. Ki67 staining, for proliferating cells, was confined to the lower third of the crypt, while expression of extracellular calcium-sensing receptor was seen in the upper third and surface epithelium. E-cadherin and β -catenin were expressed throughout the epithelium and confined to the cell surface. In tumor tissue, the same disorganized, abnormal glandular structures seen at time zero were present after 2 d. The majority of cells in these structures were mucin-poor, but occasional goblet cells were seen and mucin staining was present. Ki67 staining was seen throughout the abnormal epithelium and calcium-sensing receptor expression was weak and variable. E-cadherin was seen at the cell surface (similar to normal tissue), but in some places, there was diffuse cytoplasmic staining. Finally, intense cytoplasmic and nuclear β -catenin staining was observed in cultured neoplastic tissue.

Keywords

Colon; Organ culture; Explant; Neoplasm; Calcium

Introduction

Normal human colonic epithelium maintains a dynamic equilibrium among proliferation, differentiation, and apoptosis. Cells at the base of the colonic crypt divide rapidly (four to five times) before they begin to differentiate and move upwards in the crypt. Carcinogenesis in the colon is a complex, multistep process. Areas of dysplasia develop in the colonic mucosa and eventually some of the dysplastic cells give rise to raised tubular polyps (adenomas). Over the years, some of the raised polyps progress to invasive cancers (Burgart 2002). Most of the present understanding of colonic epithelial cell physiology and pathophysiology comes from experiments in animal models (Taketo and Edelman 2009) or from human colon-cancer-derived cell lines (Gouyer et al. 2008).

The organ culture approach allows for the study of physiology and pathophysiology at the whole tissue level. Our laboratory routinely uses skin in organ culture for that purpose. We find that skin is relatively easy to grow in culture for up to weeks, with histological and biochemical features maintained (Varani et al. 1993, 1994). Colon tissue, although possibly more complex and problematic, has also been studied in organ culture. Past studies have demonstrated that normal human colonic tissue can be maintained ex vivo for several days (Autrup 1980; Senior et al. 1982; Lipkin 1985; Moorghen et al. 1996; Kesisoglou et al. 2006). Neoplastic colonic tissue can also be sustained in culture (Kalus 1972; Kolodkin-Gal et al. 2007), although there is less experience with neoplastic tissue, and it is not known how well features unique to the neoplastic state are maintained. In the present report, we describe conditions that are suitable for organ culture of human colon tissue, including pre-malignant adenomas, invasive colon cancer, as well as normal colonic mucosa. Further, we demonstrate that histological, histochemical, and immunohistochemical markers that distinguish normal and neoplastic tissue are maintained. Organ culture fluids obtained at the end of the incubation period indicate that tissue remains metabolically active during the culture period.

Materials and Methods

Culture medium and reagents

The culture medium used here is based on a serum-free modification of earlier formulations from Kesisoglou et al. (2006), Schmiedlin-Ren et al. (1993) and Autrup et al. (1978). The basal medium consists of a mixture of 80% CMRL Medium 1066 (Invitrogen, Grand Island, NY) and 20% Hams F-12 Nutrient Mixture (Invitrogen) with: 25 mM glucose (Sigma–Aldrich, St. Louis, MO), 2 mM GlutaMax I (Invitrogen), 0.1 μ M sodium selenite (Sigma–Aldrich), 3 μ M zinc sulfate (Sigma–Aldrich), 145 nM menadione sodium bisulfate-vitamin K (M2518, Sigma–Aldrich), 45 nM(+)- α -tocopherol acetate (T1157, Sigma–Aldrich), and 50 μ g/ml gentamicin (Invitrogen). The medium is made serum-free with modifications from Moorghen et al. (1996): 3 μ g/ml hydrocortisone (H0888, Sigma–Aldrich), 50 ng/ml glucagon (G3157, Sigma–Aldrich), 0.5 ng/ml 3,3',5-triiodo-L-thyronine sodium salt (T5516, Sigma–Aldrich), 1 mg/ml bovine serum albumin (8806, Sigma–Aldrich), and 10 μ g/ml insulin from bovine pancreas (I6634, Sigma–Aldrich). We further supplement with 50 μ g/ml bovine pituitary extract (P1476, Sigma–Aldrich).

Tissue

Human colon tissue specimens (split-thickness) were obtained from surgical resections performed at the University of Michigan Hospitals. A total of nine specimens were cultured, from six females and three males, between the ages of 53 and 78 yr. The normal tissue (beyond the edge of the abnormality) and associated cancer tissue were collected from two adenocarcinomas and one sessile adenoma. Five specimens were cultured from the normal tissue only (five patients with a cancer diagnosis and one associated with Crohn's disease). The Tissue Procurement Core Laboratory at the University of Michigan Comprehensive Cancer Center was responsible for collecting the tissue at surgery and providing it to approved investigators. The study was evaluated by the institutional review board (IRB) and determined to be exempt from IRB oversight. The tissue was transported to the laboratory in cold 80% CMRL Medium 1066/20% Hams F-12 nutrient mixture, supplemented with 25 mM glucose, 2 mM GlutaMax I, 50 μ g/ml gentamicin, and 2.5 μ g/ml (1 $\times$) amphotericin (Invitrogen). The transport medium was pre-equilibrated in 5% CO₂ and 95% O₂.

Organ culture protocol

The tissue was washed three times and dissected in cold phosphate-buffered saline (Invitrogen). The mucosa and submucosa were gently teased away from the muscularis

propria, and then cut into pieces at approximately 3–4 mm³ in size. The pieces were placed, luminal-side up, on a 100 µm cell strainer (BD Biosciences, Bedford MA) which was inserted into a 35 mm² well of a 6-well dish (Corning, Corning, NY). The pieces were partially submerged by adding medium until it was approximately 1 mm above the strainer membrane (6.2 ml total volume). The dish was placed in a modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA), gassed for 20 min with 5% CO₂ and 95% O₂, and then incubated at 37°C. At 24 h, the medium was changed and the chamber was re-gassed. At the end of the 2-d incubation, medium (24 h) was collected for analysis and tissue was fixed in 10% buffered formalin for histology.

Histology and immunohistochemical markers

Zero-time control tissue from each specimen and 48 h-incubated tissue from organ culture were formalin-fixed and paraffin-embedded. Five-micrometer sections were prepared and stained with hematoxylin and eosin. In addition, tissue sections were immunostained for Ki-67, E-cadherin, and β-catenin (antibodies from Chemicon Inc.; Temecula, CA) and for calcium-sensing receptor (CaSR; antibody from Abcam Inc, Cambridge, MA). All of the antibodies were known to react with their cognate antigens in formalin-fixed, paraffin-embedded tissue. Finally, tissue sections were stained with Alcian Blue pH 2.5 (Rowley Biochemical Institute Inc., Danvers, MA) for mucopolysaccharide expression.

Matrix metalloproteinase analysis

Culture fluids were assayed for matrix metalloproteinase type I (MMP-1) by Western blotting (Varani et al. 2007) with anti-rabbit antibody to human MMP-1 (Chemicon). Briefly, samples were separated in SDS-PAGE under denaturing and reducing conditions and transferred to nitrocellulose membranes. After blocking with a 5% nonfat milk solution in Tris-buffered saline with 0.1% Tween (TTBS) at 4°C overnight, membranes were incubated for 1 h at room temperature with the antibody, diluted 1:1,000 in 5% nonfat milk/TTBS. Thereafter, the membranes were washed with TTBS and bound antibody detected using the Phototope-HRP Western blot detection kit (Cell Signaling Technology, Danvers, MA). Images were scanned, digitized and quantified.

Results

Histological/histochemical features of normal and neoplastic colon tissue in organ culture

Initially, we evaluated normal tissue from four different resections to optimize culture conditions. The specimens were from tissue beyond the edge of the abnormality (one adenoma and three adenocarcinomas). As part of these studies, a number of media formulations and culture conditions were evaluated. After 2 d in culture, the tissue was examined for structural integrity by histological analysis. The following observations were made. In medium containing fetal bovine serum, much of the normal crypt structure was lost. Epithelial cells were present in a disorganized array and few goblet cells were seen. Formulations in which the Ca²⁺ concentration was optimized for epithelial cell growth (0.15 mM) did not maintain cell viability in the stroma or in the epithelium and the tissue rapidly became completely necrotic. Growth factor-free medium was also compared with nutrient-rich medium. Our previous work has demonstrated that the optimal medium for organ culture of *skin* is a physiological calcium (1.5 mM), serum-free, growth factor-free medium; for human (Varani et al 1993) and for at least one other species, minipig (Dame et al. 2008). Growth factor-free Keratinocyte Basal Medium (Lonza, Walkersville, MD), supplemented to 1.5 mM calcium, maintained good histological structure in colon culture (data not shown), and could indeed provide a useful model for studies that require a supplement-free environment. Contrary to *skin*, though, the growth factor-supplemented medium proved superior in colon organ culture. Histological features in both the epithelial and stromal

components of the tissue were better preserved. This formulation, a serum-free, physiological calcium (1.5 mM), nutrient-rich medium, was used throughout the remainder of the experiments. Table 1 summarizes the medium components.

Finally, we adopted two common colon organ culture methods (reviewed in Resau et al. 1991): (1) maintaining the mucosa at the gas-liquid interface (Browning and Trier 1969), and (2) incubating the tissue in 5% CO₂ and 95% oxygen (Autrup et al. 1978). When the tissue was maintained fully submerged, the interstitium became edematous. Edema was much less apparent in tissue maintained with the mucosal surface at the gas-liquid interface and only the stromal surface submerged. Autrup first demonstrated with human colon the benefits of incubation under increased oxygen tension in order to control pH. We followed this procedure with 5% CO₂ and 95% oxygen. Figure 1 shows low- and high-power hematoxylin- and eosin-stained images of normal tissue after 2 d in culture under the optimized conditions as described here. Normal elongated crypt structure is evident, with goblet cells lining the lumen. The high-magnification image shows small dense cells at the base of the crypts.

Neoplastic tissue from three patients was also cultured under the same conditions. Figure 2 shows low and high magnifications of a section of a sessile adenoma. Indicative of this disease state, normal crypt structure is lost. Glandular structure is still apparent, with a variety of sizes and shapes characterizing the abnormal mucosal glands. It can also be seen that most of the goblet cells are missing. Densely packed cells with high nuclear to cytoplasmic ratio and overlapping pleomorphic cells with oblong nuclei, have replaced the goblet cells typically seen in the normal colonic crypts.

Fixed sections were also stained with Alcian Blue (Fig. 3). This stain has a strong affinity for mucopolysaccharides. Mucin production is evident in the goblets of the normal colonic crypts as well as in the luminal secretions (*a*). In the sessile adenoma (*b*), there is prolific mucin staining throughout the lumens of the abnormal glands.

Expression of growth and differentiation markers in organ-cultured colon tissue

Figure 4 shows immunostaining of normal colon tissue after 2 d in organ culture with antibodies to three different growth and differentiation markers. Ki67 (proliferation marker) is shown in panel *a*. In both the low-power and high-power images, it can be seen that staining is confined to the lower part of the crypt. Panels *b* and *c* show staining of normal tissue for expression of E-cadherin and β -catenin. In both cases, staining is predominantly located at the cell surface where there is contact between adjacent cells.

Figure 5 shows expression of the same markers in malignant colon tissue after 2 d in organ culture. Ki67 expression can be seen throughout the epithelial component of the tissue (panel *a*). E-cadherin expression is shown in panel *b*. In the majority of the tissue, its expression is cell surface in nature; not significantly different from what was observed in the normal tissue section. However, in some areas of the tissue section, cell surface expression is lost, and a diffuse intracellular staining pattern can be seen. Evidence of glandular structure is absent in these areas, and many of the cells appear to be necrotic. β -catenin staining is demonstrated in panel *c*. In contrast to what was observed in the normal tissue section (cell surface staining only), the tumor section demonstrates intense staining throughout the cytoplasm. At high power, β -catenin staining is still evident at the cell surface, but here it also can be seen in the nucleus.

Figure 6 shows CaSR staining in normal (panel *a*) and malignant tissue (panel *b*; adenocarcinoma) after 2 d in organ culture. In the normal tissue, staining is predominantly observed in the upper portion of the crypt and at the surface. The most intense staining is in

granular bodies that appear to be supra-nuclear in location. In the tumor tissue, CaSR expression is reduced in intensity and is diffuse.

MMP-1 levels in organ culture fluid

Past studies have demonstrated that many different growth factors, cytokines, matrix components, and enzymes can be quantified in serum-free organ culture fluid (Varani et al. 2007; Dame et al. 2009). We assessed MMP-1 in day 2 organ culture fluid (24 h collection) from normal and neoplastic colon tissue. As seen in Fig. 7, a higher level was observed in culture fluid from the tumor tissue.

Discussion

Our understanding of the events that regulate growth and differentiation in the colon come largely from studies in experimental animals (Taketo and Edelman 2009) or from studies with human colon carcinoma cells in vitro (Gouyer et al. 2008). The use of organ culture technology provides an alternative approach with which to address questions of physiology and pathophysiology. Successful organ culture requires conditions that not only maintain cell viability but also preserve features expressed in the intact tissue. In the present study, we have identified conditions that allow for the maintenance of human colon tissue in organ culture and the preservation of histological, histochemical and immunohistochemical features that distinguish normal and neoplastic colon tissue in vivo. For example, in normal tissue, crypt histology was indistinguishable from that seen in fresh tissue. Proliferation, as assessed by Ki67 staining, was confined to the lower third of the crypt; CaSR expression was seen in the upper part of the crypt and at the surface; and E-cadherin and β -catenin were expressed at the cell surface between adjacent cells. Staining with Alcian Blue (mucopolysaccharide) was confined to goblet cells and to the material secreted into the crypt lumens from goblet cells. In contrast to this, in malignant tissue, Ki67 staining was observed throughout the epithelium, while CaSR expression was diffuse or absent altogether. β -catenin was expressed throughout the cytoplasm and nucleus. E-cadherin was largely expressed at the cell surface (similar to what was observed in the normal tissue sections) but in some areas, diffuse expression was seen throughout the cytoplasm. Where this was observed, all glandular structure was lost and many of the cells appeared to be necrotic. As was seen with normal tissue sections, Alcian blue staining was observed wherever goblet cells remained, and their secretory products were evident.

The ability to maintain histological and immunohistological features of normal and neoplastic colon tissue in organ culture provides a novel system to address some of the unresolved questions in colon cancer biology as well as questions pertinent to other colon diseases. One of our primary interests is the role of Ca^{2+} /CaSR in regulation of growth and differentiation. We know from immunohistochemical studies of fresh colon tissue that CaSR expression in neoplastic cells is reduced as compared with normal colonic mucosa, or lost completely (Chakrabarty et al. 2003, 2005). Studies with isolated colon carcinoma cells in monolayer culture have demonstrated that some cells do not respond to extracellular Ca^{2+} with reduced growth and the onset of differentiation. Loss of Ca^{2+} sensitivity is associated with reduced CaSR expression (Bhagavathula et al. 2005, 2007). While these cells are resistant to the growth-inhibiting effects of physiological Ca^{2+} , some of the cells undergo differentiation when the level of extracellular Ca^{2+} is raised approximately fourfold (i.e., to 5.6 mM; Aslam et al. 2009). This provides an explanation at the cellular level for the benefits of calcium supplementation for colon cancer chemoprevention (Baron et al. 1999). One can envision using intact (ex vivo) colon tissue to help elucidate the signaling events that occur when normal tissue is exposed to elevated concentrations of Ca^{2+} , and further, to examine what occurs differently in pre-malignant or malignant tissue.

Short-term organ culture of colon might especially be useful for the study of these cellular events, as the life cycle of colonic basal cells is a few days. Here, the stem cells either progress through transit amplifying progeny and ultimately to differentiation, or lose their ability to differentiate, continue cycling, and initiate carcinogenesis. The fate of the basal cell is intricately associated with the crypt milieu (Whitfield 2009). The culture of whole tissue allows for experimental manipulation of the crypt environment and timely observations. This would be problematic in the animal model and possibly over simplified at the level of cell culture.

Because of its inherent similarity to in situ conditions, other opportunities for use of human colon tissue in organ culture should be found. The ultimate utility of this approach, however, will depend on a number of factors. First, and foremost, is the degree to which findings in organ culture mimic what is seen in vivo. The histological, histochemical, and immunohistochemical findings presented here suggest that the relationship between in vivo and ex vivo findings is close. It must be emphasized, however, that to date only a limited comparison has been made. Additional work will be required before we can fully appreciate how findings in the organ culture model mimic what occurs in vivo.

Organ culture use is also dependent on tissue availability. Fortunately (or otherwise), resection of the large bowel occurs frequently for treatment of cancer and other conditions. While certainly not as readily available as skin, sufficient numbers of colon surgeries are carried out that one can plan a study that relies on this tissue. Associated with availability is ease of use. Our laboratory has over the years made extensive use of skin in organ culture, both human (Varani et al. 1993, 1994, 2007) and minipig (Dame et al. 2008, 2009). Skin is readily available and the culture conditions needed for maintenance are simple. Typically, 2-mm-size pieces of skin are grown in serum-free, growth factor-free medium under fully submerged culture conditions at 37°C and 5% CO₂ and 95% air. Conditions needed for optimal maintenance of human colon in organ culture appear to be somewhat more stringent. In contrast to the serum-free, growth factor-free medium, which is optimal for skin, a culture medium enriched with a number of growth factors is necessary for optimal preservation of colon tissue features. Another requirement for colon organ culture is the presence of high oxygen tension. Whereas skin is adequately maintained in 5% CO₂/95% air, colon is best preserved in 5% CO₂/95% O₂. The high metabolic rate of the colonic epithelium may account for the increased oxygen requirement. Alternatively, gas permeation to the proliferating epithelial cells at the base of the crypts may be less efficient than it is to the basal keratinocytes in organ-cultured skin. The fact that slightly larger tissue pieces are used with colon than typically used in skin cultures may also contribute to decreased gas permeation. Another important difference between skin and colon organ culture is the use of an air-liquid interface. The orientation of the tissue with the mucosal surface at the air interface proved important. The reason for this is unclear, but we observed that submerged mucosal tissue became edematous.

In Conclusion

1. A number of media formulations were evaluated for preservation of colon structure/function in organ culture. A medium rich in growth factors was beneficial for maintenance of mucosal integrity. Fetal bovine serum was not helpful, but as with skin, a physiological calcium concentration (1.5 mM) was required for preservation of tissue.
2. Optimal culture conditions included 5% CO₂ and 95% O₂ with the mucosa facing the gas interface and the connective tissue partially submerged. When fully submerged, the tissue became edematous.

3. Histological, histochemical, and immunohistochemical features indicative of in situ characteristics of normal and neoplastic colon tissue were preserved.

Based on these findings, we conclude that human colon tissue can be maintained in organ culture, with preservation of structure and function unique to normal and neoplastic tissue. This model may provide a valuable tool to manipulate and study normal vs. neoplastic states at the whole tissue level.

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Figure 1. Histological features of normal colon tissue in organ culture. Formalin-fixed tissue is hematoxylin- and eosin-stained. Normal colon crypt structure is maintained after 2 d in culture. Original magnification *left panel*, $\times 125$; *right panel*, $\times 340$.

Figure 2. Histological features of neoplastic colon tissue in organ culture. Formalin-fixed tissue is hematoxylin- and eosin-stained. Tumor component of the tissue (sessile adenoma) is preserved after 2 d in culture. Original magnification *left panel*, $\times 125$; *right panel*, $\times 340$.

Figure 3. Alcian Blue staining of normal and neoplastic colon tissue in organ culture. Normal (a) and a sessile adenoma (b) continue to express mucin after 2 d in culture. Original magnification, $\times 300$.

Figure 4.

Immunoperoxidase staining of normal tissue with antibodies to growth and differentiation markers after 2 d in organ culture. *a*, Ki67: intense staining is observed in rapidly growing cells at the base of the crypt. As the cells move upward in the crypt, cells start to differentiate and little or no staining for Ki67 is seen in these regions. *b*, E-cadherin: a marker of differentiation and present at cell to cell contacts. Well-defined membrane staining of E-cadherin is observed. Intensity of E-cadherin staining progressively increases towards upper regions of the tissue where more differentiated cells are present. *c*, β -catenin: well-defined membrane staining of β -catenin is observed in normal tissue. Little or no nuclear staining of β -catenin is seen. Nuclear β -catenin staining would be an indicator of uncontrolled proliferation of epithelial cells. Original magnification *left panel*, $\times 170$; *right panel*, $\times 540$.

Figure 5. Immunoperoxidase staining of malignant colon tissue after 2 d in organ culture (adenocarcinoma). *a*, Ki-67: Intense staining, indicating proliferation, is seen throughout the section. *b*, E-Cadherin: Junctional and intracellular staining is observed. Cells in some areas of the section have little or no membrane staining, with diffuse intracellular staining—a marker of tumor malignancy. *c*, β -catenin: strong cytoplasmic and nuclear staining is evident. The distinct membrane staining is lost in places. At higher magnification, nuclear staining can be seen (*bottom panel, arrows*). Original magnification *left panel*, $\times 170$; *right panel*, $\times 540$.

Figure 6. Calcium-sensing receptor staining of normal and malignant colon tissue in organ culture. After 2 d in organ culture, normal tissue *a* expresses CaSR at in the upper part of the crypt and the malignant tissue *b* (adenocarcinoma) exhibits diffuse staining with reduced intensity. Original magnification, $\times 320$.

Figure 7. Matrix metalloproteinase-1 elaboration (*MMP-1*; interstitial collagenase). 24 h culture supernatant fluids were collected on day 2 and analyzed for MMP-1 by Western blot. A higher level of MMP-1 was present in culture fluid from tumor compared with the normal tissue. The figure represents averages $\pm$ ranges of two independent experiments.

Table 1

Colon organ culture media

	Final concentration
Basal components	
CMRL medium 1066	80%
Hams F-12 nutrient mixture	20%
Glucose	25 mM
GlutaMax I	2 mM
Gentamicin	50 µg/ml
Sodium selenite	0.1 µM
Zinc sulfate	3 µM
Supplements and growth factors	
+)-α-tocopherol acetate	45 nM
3,3',5-triiodo-L-thyronine sodium salt	0.5 ng/ml
Menadione sodium bisulfate	145 nM
Glucagon	50 ng/ml
Hydrocortisone	3 µg/ml
Insulin from bovine pancreas	10 µg/ml
Bovine serum albumin	1 mg/ml
Bovine pituitary extract	50 µg/ml