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MORPHOLOGIC DIFFERENTIATION OF COLON CARCINOMA CELL LINES HT-29 AND HT-29KM IN ROTATING-WALL VESSELS

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SUMMARY

A new low shear stress microcarrier culture system has been developed at NASA's Johnson Space Center that permits three-dimensional tissue culture. Two established human colon adenocarcinoma cell lines, HT-29, an undifferentiated, and HT-29KM, a stable, moderately differentiated subline of HT-29, were grown in new tissue culture bioreactors called Rotating-Wall Vessels (RWVs). RWVs are used in conjunction with multicellular cocultivation to develop a unique in vitro tissue modeling system. Cells were cultivated on Cytodex-3 microcarrier beads, with and without mixed normal human colonic fibroblasts, which served as the mesenchymal layer. Culture of the tumor lines in the absence of fibroblasts produced spheroidlike growth and minimal differentiation. In contrast, when tumor lines were co-cultivated with normal colonic fibroblasts, initial growth was confined to the fibroblast population until the microcarriers were covered. The tumor cells then commenced proliferation at an accelerated rate, organizing themselves into three-dimensional tissue masses that achieved 1.0- to 1.5-cm diameters. The masses displayed glandular structures, apical and internal glandular microvilli, tight intercellular junctions, desmosomes, cellular polarity, sinusoid development, internalized mucin, and structural organization akin to normal colon crypt development. Differentiated samples were subjected to transmission and scanning electron microscopy and histologic analysis, revealing embryoniclike mesenchymal cells lining the areas around the growth matrices. Necrosis was minimal throughout the tissue masses. These data suggest that the RWV affords a new model for investigation and isolation of growth, regulatory, and structural processes within neoplastic and normal tissue.

Key words: rotating-wall vessels; tissue modeling; microcarrier; three-dimensional; differentiation; colon cancer.

INTRODUCTION

An important principle of cellular physiology is the interaction of multiple cell types and their association with cellular differentiation. As predicted by embryologists for more than 50 yr, cellular differentiation is effected and maintained by complex cellular interactions (14,42,44). Mechanisms for these cellular interactions involve cell membrane junctions, extracellular matrices (e.g., basement membrane and ground substances), and soluble signals (autocrine, paracrine, and endocrine). In human endometrium, isolated epithelium is not responsive to estrogen in spite of the presence of estrogen receptors (24), presumably due to a lack of other tissues necessary for the proliferative response observed in vivo. Although cellular differentiation is genetically based, the process is also influenced by the cellular environment, particularly the three-dimensional spatial relationship of cells to each other and to extracellular matrices and factors (6,9,10,20).

For long-term culture, the best results are obtained in epithelial cells cultured in association with a fibroblast or endothelial "feeder" layer. In hepatocytes, for example, this configuration improves both culture longevity and tissue-specific functions such as hydrocortisone-inducible tyrosine aminotransferase. Co-culture of rat hepatocytes with endothelium and Kupffer cells resulted in greater retention of the ultrastructural markers that distinguish these cell types. Furthermore, conditioned medium from a "feeder" cell line did not replace the need to co-cultivate with an additional cell type (15,33).

Present in vitro culture technology does not permit reproducible cultures of normal or neoplastic colonic epithelium in large-scale, three-dimensional configurations (7). As a consequence, the factors that control proliferation and differentiation in the gastrointestinal tract remain largely unknown (1–3,8,27,32). Culture of intestinal epithelial cells by standard techniques has limited success. One of the first techniques, organ culture, in which slices of tissue were placed in culture medium, permitted only short-term analysis of crypt population dynamics. Long-term cultures were difficult to achieve (longer than 7 wk) because crypt cells are unable to survive standard culture regimens (36). Unfortunately, two-dimensional organ culture does not support the assembly of the stroma and its contribution to the growth of epithelial cells. As a result, investigators resorted to the culture of normal gut-lining epithelial cells in monolayer cultures. While short-term cultures are possible, long-term growth has required transformation with a tumor promoter, oncogenic virus, or culture in highly defined, exotic media often resulting in the loss of cellular organization and function (26). Short-term cultures of normal colon cells developed an oriented monolayer with an absorptive apical surface, junctional complexes,

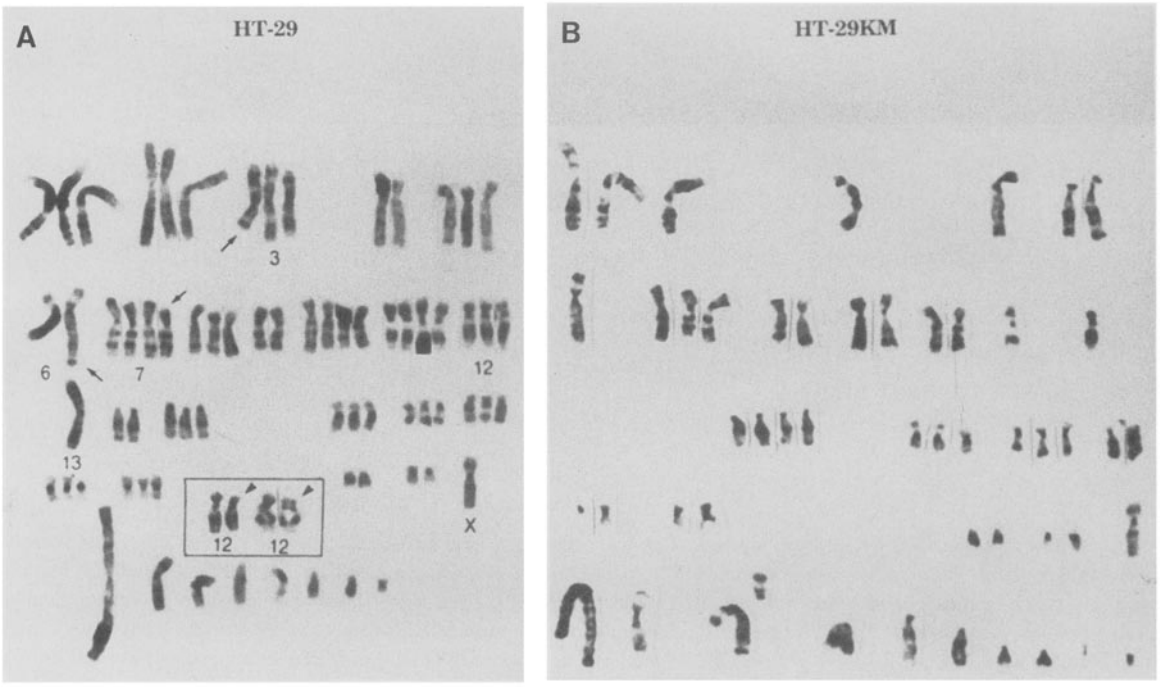


FIG. 1. Karyotypic analysis of HT-29 (*A*) and HT-29KM (*B*).

and dome formation. In addition, overlays of basement membrane preparations aided in the differentiation of some cultures of neoplastic colonic epithelium (5,21–23). The culture of primary human large bowel neoplastic cells, which have less stringent growth requirements than normal colonic epithelium, has a low success rate. Only 18 to 29% of adenocarcinomas of the colon or rectum can be established in vitro. In comparison, 62% of colorectal carcinomas may be established as xenografts in athymic nude mice (19). While not all human carcinomas propagate in immunocompromised mice, more carcinomas grow in nude mice than in current tissue culture systems. Although animal models are useful for certain studies, many biochemical and molecular studies require that cells be grown in vitro. Due to the lack of a successful long-term technology to culture gut epithelium, a new technology developed at NASA's Johnson Space Center is now being used to construct a large-scale, three-dimensional in vitro tissue culture model which compares favorably with the nude mouse model. Briefly, Rotating-Wall Vessels (RWVs) are horizontally rotating cylindrical tissue culture vessels that provide controlled supplies of oxygen and nutrients, with minimal turbulence and extremely low shear (45). These vessels suspend cells and microcarriers homogeneously in a nutrient-rich environment which allows the three-dimensional assembly of cells to tissue.

MATERIALS AND METHODS

Cell lines. Two colon adenocarcinoma cell lines were used: HT-29, an undifferentiated adenocarcinoma originally isolated by J. Fogh at the Walker Laboratory, Rye, NY (13) and HT-29KM, a moderately differentiated subline of HT-29 isolated by passage through athymic BALB/c nude mice. Karyotypic analysis of the two lines demonstrates that HT-29 has a hyperdiploid complement of chromosomes (Fig. 1 *A*) whereas HT-29KM is near diploid but contains several marker chromosomes of HT-29 (Fig. 1 *B*). Further, HT-29 grows as an undifferentiated tumor xenograft that lacks gland formation (Fig. 2 *A*) whereas HT-29KM is a moderately differentiated xenograft that forms glands and mucin-containing signet ring cells (Fig. 2 *C*). Normal adult colon fibroblasts were established from primary cultures of the normal colons of organ donors (19). Cellular purity was established by vimentin positive AE1/AE3 and Factor VIII antibody negative immunocytochemistry. The normal fibroblast cell line CCD-112CoN and our HT-29 cultures were obtained from the American Type Culture Collection (Rockville, MD).

Monolayer culture. All cells were grown in monolayer culture in a Forma Stericult 100 humidified CO_2 incubator. The cultures were maintained in a 95% air:5% CO_2 constant atmosphere at a temperature of 37° C. Growth medium for all cultures was minimal essential medium Alpha (MEM α) with 100 mg/dl glucose (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT); 1% nonessential amino acids, 1% MEM vitamins, 2 g/liter HEPES, and 1% L-Glutamine, 100 U penicillin, 100 μg streptomycin, and 2.5 μg/ml Fungizone (GIBCO); and 2.5 μg/ml insulin, 2.5 μg/ml human transferrin, and 2.5 ng/ml sodium selenite (Sigma Chemical Co., St. Louis, MO).

Cells were passaged as required by enzymatic dissociation with a solution of 0.1% trypsin, 0.1% EDTA, for 15 min. After incubation with the appropriate enzymes, the cells were centrifuged at 800 *g* for 10 min in Corning Conical 15-ml centrifuge tubes. The cells were then resuspended in fresh medium and diluted into Corning T-75 flasks with 25 ml of fresh growth medium.

Monolayer controls for RWV experiments. Samples from the cellular inoculum for each RWV experiment (2×10^5 cells/ml) were placed in Corning T-75 flasks to serve as growth controls. For co-cultivation experiments the two cell types were mixed in a predetermined ratio and placed in culture flasks and in Dispo nonadherent petri dishes (100 × 15 mm) (Lab-Tek Division of Miles Laboratories, Naperville, IL) with microcarrier beads. The progress of control cultures was monitored and recorded photographically on a Nikon Diaphot inverted microscope equipped with Hoffman modulation optics.

Rotating-wall vessel cultures. The RWV is a horizontally rotated, zero headspace, center oxygenation, transparent culture vessel. Cells to be cul-

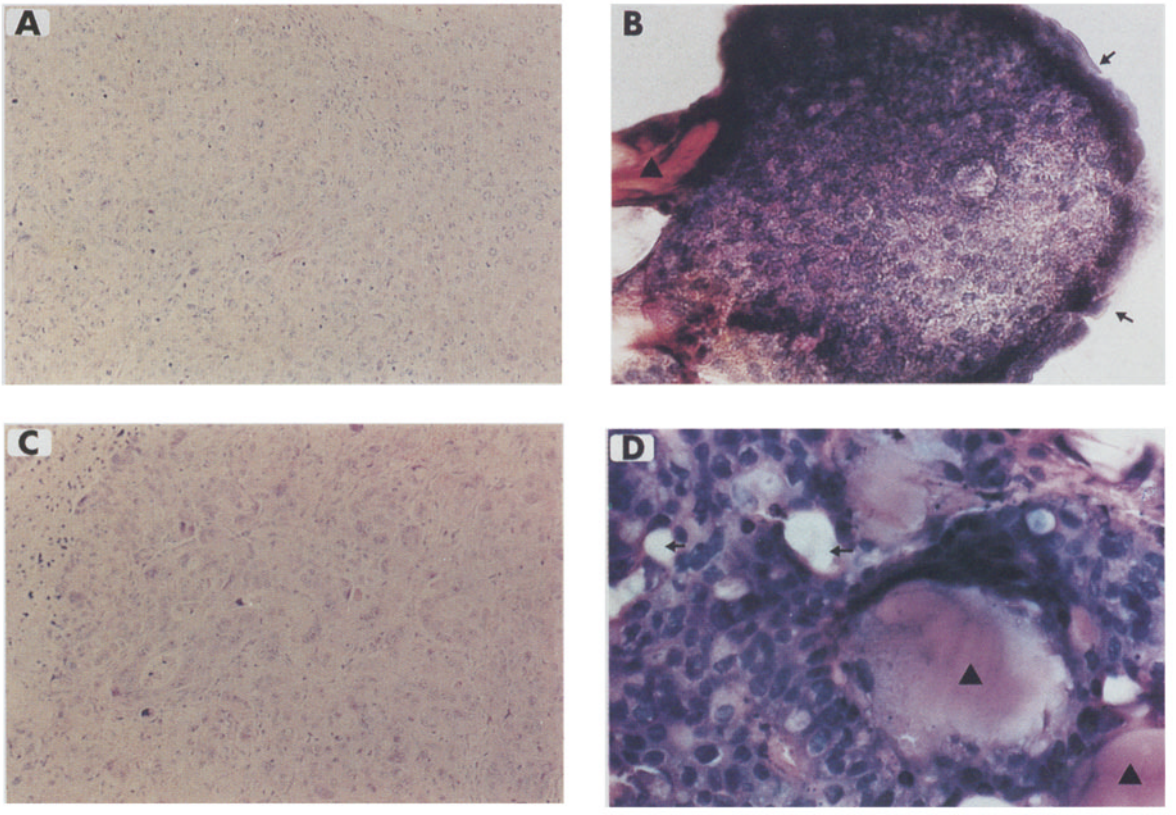


FIG. 2. Histologic comparison of mouse xenografts of HT-29 and HT-29KM vs. HT-29 and HT-29KM co-cultivated in RWVs; 5 μm H&E-stained cross sections of HT-29 (*A*) and HT-29KM (*C*) mouse xenografts, ×100. HT-29 shows no differentiation and HT-29KM shows areas of limited cellular differentiation and signet ring cell development (*lower left*); 10 μm H&E-stained co-cultures of HT-29 (*B*) and HT-29KM (*D*), ×200. *B*, a large polypoid growing out from the microcarrier (*far left, triangle*). *Arrows* mark the apex of the polypoid. *D*, signet ring cell formations (*arrows*). In contrast to *C*, the tissue in *D* shows structural organization and differentiation. Multiple signet ring cells and glandular formations are visible. In addition, a line of columnar epithelial cells can be seen to the immediate left of the microcarrier in the center of the photograph (*D*).

tured in the RWV were grown initially in T-flasks as described above in preparation for seeding into the vessels. The initial inoculum for each experiment was 2×10^5 cells/ml, with 5 mg/ml Cytodex-3 microcarriers (Pharmacia, Piscataway, NJ) which resulted in a starting concentration of 10 cells per bead. Cytodex-3 microcarriers are type 1, collagen-coated dextran beads of 175 μm in diameter. The concentration of beads was maintained at 5 mg/ml. Co-cultivation experiments used 10% tumor cells and 90% fibroblasts as the starting inoculum.

Tumor cells and fibroblasts were removed from T-75 flasks by enzymatic digestion, washed once with calcium- and magnesium-free phosphate buffered saline (PBS), and assayed for viability by trypan blue dye exclusion (GIBCO). Cell types were mixed in the proper ratio and placed in growth medium on ice before inoculation. After inoculating the RWV, the cultures were allowed to grow for 48 h before the medium was changed. Thereafter, the medium was changed every 20 to 24 h; as the metabolic requirements of the cultures increased, fresh medium was supplemented with an additional 100 to 200 mg/dl of glucose. The growth curves for each experiment were reported as average number of cells per milliliter vs. incubation time.

Histologic analysis. Samples from RWV cultures were taken at multiple time points throughout the course of the experiments for histologic analysis. Two basic stains were performed on the 20, 10, and 6-μm sample sections, hematoxylin and eosin (H&E) and mucicarmine staining. After removal from the reactor vessels, samples were washed once with calcium- and magnesium-free PBS. The samples were suspended in a buffer containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 *M* cacodylate buffer at pH 7.4 and analyzed by histology and electron microscopy (25).

Mucicarmine staining was performed according to the procedure of Sheehan and Hrapchak (37). Tissues were fixed as previously stated. The samples were embedded in paraffin, blocked, and cut in multiple thickness as stated above. Sections were then mounted on slides and deparaffinized. The preparations were stained with Weigert's iron hematoxylin working solution for 5 min, then rinsed with running tap water for 5 min and placed in mucicarmine working solution for 30 min at room temperature. Slides were then rinsed with deionized water, stained with tartrazine solution for 1 to 5 s, then rinsed again with deionized water. The slides were dehydrated with xylene and mounted for analysis.

Scanning (SEM) and transmission electron microscopy (TEM). Samples from the RWV cultures were taken for SEM at the same times as those taken for histologic analysis. Samples were fixed as described above, then rinsed for 5 min with cacodylate buffer 3 times and postfixed with 1% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA) in cacodylate buffer for 1 h. Samples were then rinsed for 5 min with distilled water 3 times and then treated for 10 min with Millipore (Millipore Corp., Bedford, MA) (0.2 μm) filtered saturated solution of thiocarbohydrazide (Electron Microscopy Sciences), then washed for 5 min with distilled water 5 times and fixed with 1% buffered osmium tetroxide for 10 min. This last step was necessary to prevent the beads from collapsing. Samples were then rinsed with distilled water 3 times and dehydrated with increasing concentrations of ethanol followed by three changes in absolute methanol, and divided for TEM and SEM.

Samples for SEM were transferred to 1,1,1,3,3,3-hexamethyldisilazane

TABLE 1

MAXIMUM CELL NUMBERS IN DIFFERENT CULTURE CONDITIONS[a]

Condition	HT-29	HT-29KM	Normal Colon Fibroblasts
1. Monoculture in nonadherent dishes	NG[b]	NG[b]	NG[b]
2. Monoculture in nonadherent dishes on microcarriers	$\approx 1.0 \times 10^6$	$\approx 1.0 \times 10^6$	$\approx 0.3 \times 10^6$
3. Monocultures in RWVs on microcarriers	2.7×10^6	3.3×10^6	0.5×10^6
4. Coculture in RWVs on microcarriers with normal colon fibroblasts	9.3×10^6	10.9×10^6	—

[a] Number of cells/ml. [b] NG = no growth.

(HMDS) (Electron Microscopy Sciences) where they were allowed to soak for 10 min. Samples were then drained and allowed to air dry overnight. Dried samples were sprinkled with a thin layer of silver paint on a specimen stub. The paint was allowed to dry and the samples were coated by vacuum evaporation with platinum-palladium alloy. The samples were then examined in the AMRAY 1000 SEM at an accelerating voltage of 5 to 10 kV.

Samples for TEM were fixed as were all other samples in 3% gluteraldehyde, 2% paraformaldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4. They were washed in 0.1 *M* cacodylate buffer (3 times for 10 min), postfixed in 1% osmium tetroxide in 0.1 *M* cacodylate buffer and washed in 0.1 *M* cacodylate buffer (3 times for 10 min). Specimens were dehydrated in an ascending series of acetone (30, 50, 70, 95, and 2 × 100%, 5 min each), transferred to propylene oxide (twice for 10 min) and infiltrated in Spurr low viscosity embedding medium. The infiltration schedule consisted of increasing ratios of Spurr resin: propylene oxide (1:1, 1 h; 2:1, 2 h; and pure resin, 4 h). Individual pieces, approximately 2 to 4 mm^3, were transferred to silicone flat embedding molds containing fresh Spurr resin, and the resin was polymerized at 60° C for 24 h. Thin sections (60 to 80 nm) were cut using a diamond knife on a Reichert Ultracut E ultramicrotome. The sections were collected onto naked 300 hexagonal mesh grids and were stained with saturated aqueous uranyl acetate and Reynolds lead citrate. The sections were examined and photographed using a Philips CM12 TEM operating at 80 kV.

Quantitation of morphologic structure. The number of polyps and glands and the size of tissue masses were quantitated from 5-ml samples extracted from the RWV at the conclusion of each co-culture experiment. Tissue masses were placed in 35-mm petri dishes (Limbro) with 2-mm grids and quantitated with an inverted Nikon Diaphot microscope equipped with phase contrast optics.

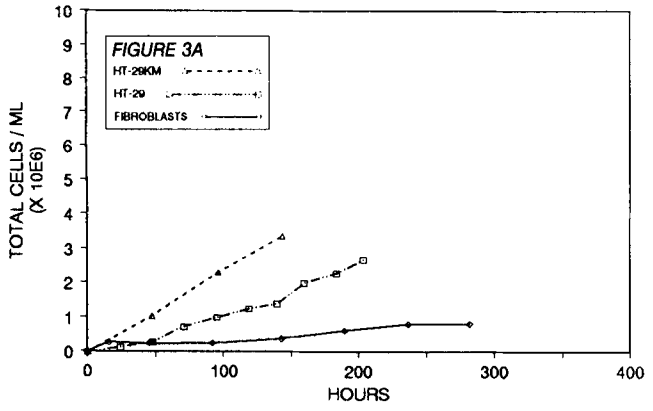
Results

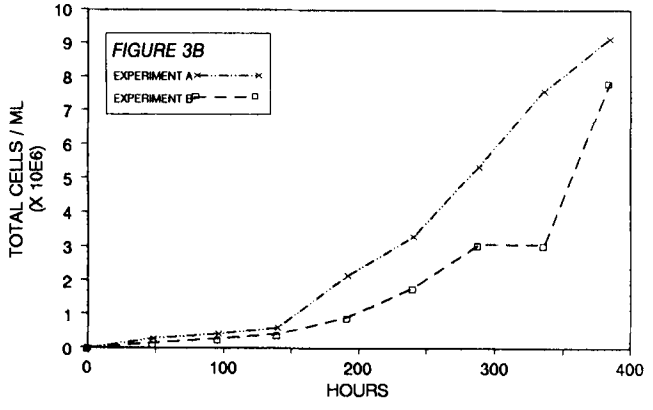
Cultures in nonadherent petri dishes. HT-29, HT-29KM, and primary normal colon fibroblasts were cultured in nonadherent petri dishes with and without microcarrier beads. When each of the three cell types was plated without microcarrier beads, clumps of undifferentiated cells aggregated and died over a 96-h period. Low cell yields were obtained when HT-29 and HT-29KM were cultured separately, each with microcarrier beads (12). Tumor cells attached to the beads and formed undifferentiated masses of cells, eventually achieving only 1.0×10^6 cells/ml. When primary normal colon fibroblasts were cultured with microcarrier beads, the cells covered the beads and rafted some beads together, finally becoming contact-inhibited, but left many bare beads. These cultures (Table 1) only reached a cell density of 0.3×10^6 cells/ml.

Monocultures in RWVs. When HT-29 or HT-29KM was cultured in RWVs on microcarrier beads, the starting inoculum was 2.0×10^5 cells/ml, and the tumor cells achieved culture-densities of approximately 2 to 4×10^6 cells/ml (Fig. 3 *A*) and grew as undifferentiated aggregates between beads (Fig. 4 *C–F*) The population doubling time for HT-29 increased from 24 h in monolayer cultures to nearly 40 h in the RWV; the doubling time for HT-29KM was extended from 36 to 61 h. Cell aggregates reached approximately 0.3 to 0.5-cm diameters with minimal necrosis. When colonic fibroblasts were cultured in the RWVs they covered and rafted the beads (Fig. 4 *A,B*). The fibroblasts grew slowly and became contact-inhibited, attaining a concentration of only 0.5×10^6 cells/ml and were maintained at confluence for 5 days (Fig. 3 *A*). When each cell type was examined for evidence of differentiation, the fibroblasts seemed similar to the connective tissue that appears in vivo (Figs. 5 *A* and 6 *A*). HT-29 seemed to form undifferentiated masses of cells (Figs. 4 *C*, 5 *B*, 6 *B*, 7 *A–C*), and HT-29KM also seemed largely undifferentiated with occasional intracellular glands but no signet ring cells, cellular polarity, columnar development, or apical microvilli (Figs. 4 *E*, 5 *D*, 6 *D*, 7 *D–F*).

Cocultivation in RWVs. HT-29 and HT-29KM were also cocultivated with normal human colonic fibroblasts, and the starting inoculum for each co-culture was 2.0×10^5 cells/ml consisting of a 9:1 ratio of fibroblasts to tumor cells (22,34). Each tumor line attained higher cell densities when co-cultured than when cultured alone: the HT-29/Fibro co-culture attained 8.0 to 9.3×10^6 cells/ml (Fig. 3 *B*), and the HT-29KM/Fibro co-culture reached 5.5×10^6 cells/ml (Fig. 3 *C*).

More importantly, the co-cultures exhibited several characteristics of morphologic differentiation (7). In the HT-29/Fibro co-cultures, tumor cells grew over fibroblast-covered beads and formed polypoid structures (Figs. 5 *C* and 6 *C*). However, these co-cultures did not produce mucus-containing signet ring cells (Fig. 5 *C*, 6 *C*, 8 *E,F*, and 2 *B*). In contrast, the HT-29KM/Fibro co-cultures exhibited enhanced morphologic differentiation and organization of polarity (31). Similar to HT-29/Fibro co-cultures, HT-29KM cells grew as polyps over fibroblasts on microcarrier beads. However, unlike HT-29, HT-29KM formed glandular "lattice" structures as visualized with SEM (Fig. 9 *C,D,*) and histology. These cultures also formed organized epithelium (Fig. 10 *A–C*) with apical microvilli, columnar epithelium (Fig. 10 *D*), large amounts of extracellular matrix material (Fig. 10 *E*), and interglandular structures (Fig. 10 *F*) which contained mucinous material as verified by mucicarmine stains (Fig. 6 *E*). It is unlikely that these structures are artifacts caused by microcarrier bead impressions, because these glands were surrounded by fibroblasts (Fig. 9 *F*) and looked very similar to a moderately differentiated colon carcinoma in vivo. Finally, signet





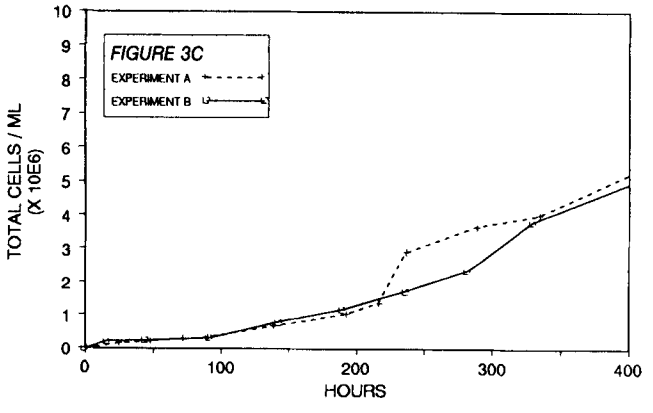


FIG. 3. *A*, growth curves of normal colon fibroblasts, HT-29, and HT-29KM grown on microcarriers in RWV. *B*, HT-29 co-cultivated with normal colon fibroblasts on microcarriers in RWV. *C*, HT-29KM co-cultivated with normal colon fibroblasts on microcarriers in RWV.

ring cells were identified (28,30) in the HT-29KM/Fibro co-cultures that were not present in either HT-29KM alone or in HT-29/Fibro co-cultures (Fig. 5 *E*, 6 *E*, 8 *E*, and 2 *D*). The appearance of signet ring cells and glands in the HT-29KM/Fibro co-cultures is similar to the appearance of HT-29KM grown in nude mice.

The number of polyps, glands, and tissue masses of different sizes was quantitated in 5-ml aliquots of medium harvested from RWVs that were plated in 35-mm petri dishes with a 2-mm grid. HT-29/Fibro co-cultures averaged 18 polyps/5 ml, 15 to 19 days after initiation of culture (Fig. 11 *A*). HT-29KM/Fibro co-cultures had slightly fewer polyps but formed an average of 35 glands/5 ml of medium harvested (Fig. 11 *A*). HT-29/Fibro co-cultures produced an average of 45 tissue masses per 5-ml sample, 32 that were 1 to 3 mm in diameter, and 13 that were more than 3 mm in diameter (Fig. 11 *B*). In contrast, HT-29KM/Fibro co-cultures formed tissue masses that were all greater than 3 mm in diameter (Fig. 11 *B*). Inasmuch as the internal diameter of the collection port was 5 mm, the size of larger tissue masses was estimated with a centimeter ruler before removal from the vessels.

DISCUSSION

The ability to construct a large-scale, three-dimensional, in vitro differentiation model affords a vast array of possibilities for cell biological investigations. The differentiation evidenced in HT-29 and especially HT-29KM co-cultures suggests that many aspects of differentiation may be displayed, under proper conditions, in otherwise undifferentiated adenocarcinoma cell lines. The presence of a) cellular organization and ordering of tissue, b) development of glandular structures, c) appearance of cellular morphologies not previously observed in the initial inoculum (signet, goblet, and columnar), and d) production of secretory product (mucin) and apical microvilli and polypoid formations collectively signify a multiphase differentiation process that is the product of the cellular components and physical orientations afforded by the culture conditions. Furthermore, differentiation is achieved without the addition of traditional inducing agents but rather is controlled by the nature of the cell populations.

To facilitate differentiation in a manner representative of the in vivo environment, without changing the base sugar of the growth medium (29,43) or using chemical induction (11,28), HT-29 and HT-29KM were co-cultivated with mixed normal human colon fibroblasts in RWVs. Our HT-29 cell line did not differentiate when it was implanted in nude mice. HT-29 cultured with normal human colon fibroblasts in RWVs exhibited organization and polarity because it grew over fibroblasts that, for the most part, remained closely attached to the microcarrier bead surface. As the polypoid masses grew away from the fibroblast surface, they broke off and formed spheroids. Furthermore, our HT-29 cell line did not produce signet ring cells in either the RWV or in the nude mouse, and thus our HT-29 line may be different from that used by Richman and Bodmer (34); HT-29KM, however, demonstrated epithelial polarity and cellular organization, and produced glands and signet ring cells when cultured in the RWV with mesenchymal cells and in vivo in the nude mouse. Thus, we conclude that the RWVs support the development of tissues that are remarkably similar to structures that are formed in vivo.

The most notable result indicates enhanced growth of neoplastic colonic epithelium in the presence of mixed normal human colonic fibroblasts in this culture system. Extensive studies performed over the last 10 yr have confirmed the capability of normal gut mesenchymal cells to induce differentiation of normal gut endoderm and epithelium in several mammalian systems (16,17,23,40). Kedinger

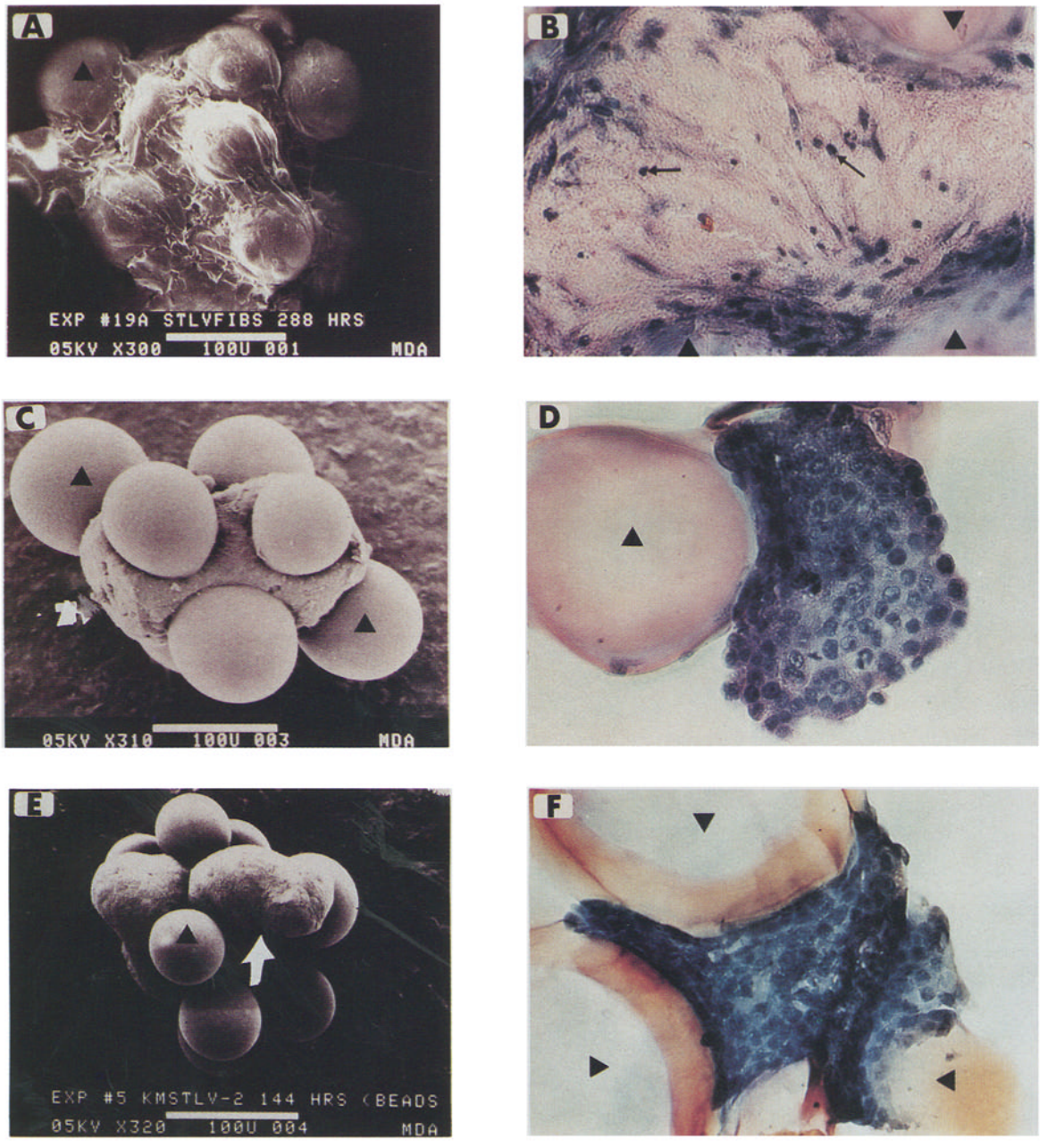


FIG. 4. SEM and histologic comparison of normal colon fibroblasts, HT-29, and HT-29KM in RWVs. SEMs of normal colon fibroblasts, ×300 (*A*), HT-29, ×310 (*C*), and HT-29KM, ×320 (*E*). *A,C,E*, tissue masses (*arrows*) grown on microcarriers (*triangles*) in RWVs; 10 μm histologic cross sections of microcarrier bead packs stained with H&E (*B,D*) or mucicarmine (*F*). Each figure shows a cell mass of normal colon fibroblasts (*B*), HT-29 (*D*), or HT-29KM (*F*) growing on or between microcarriers, ×200.

et al. (23) and Haffen et al. (16) shown that both fetal endodermal and crypt cells can be induced to differentiate when cultured in association with fetal mesenchyme followed by grafting into rats or nude mice. Differentiation in these cases included the development of villi and crypts and the appearance of columnar, goblet (mucin-producing), endocrine, and paneth cells. Levels of brush border enzyme production (i.e. sucrase and isomaltase), however, were reduced in recombinants as compared to nondissociated grafts (23). The reduced levels of enzyme production are thought to be due in part to the absence of specific extracellular matrix components which after dissociation must be synthesized de novo. Additional findings have shown that not only does the mesenchyme induce differentiation of the endoderm and epithelium, it also undergoes differentiation into connective tissues and muscle layers as a result

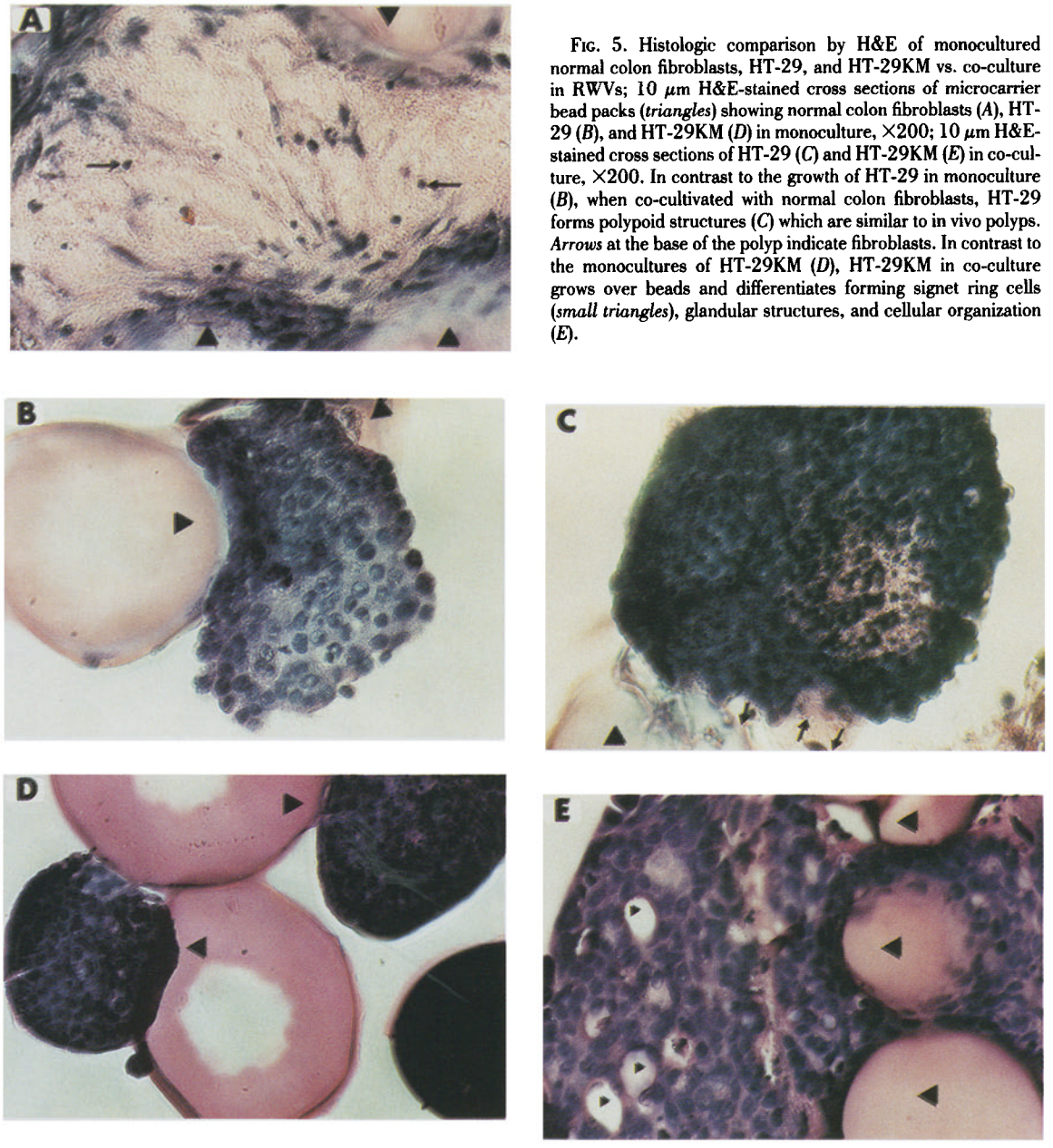


FIG. 5. Histologic comparison by H&E of monocultured normal colon fibroblasts, HT-29, and HT-29KM vs. co-culture in RWVs; 10 μm H&E-stained cross sections of microcarrier bead packs (*triangles*) showing normal colon fibroblasts (*A*), HT-29 (*B*), and HT-29KM (*D*) in monoculture, ×200; 10 μm H&E-stained cross sections of HT-29 (*C*) and HT-29KM (*E*) in co-culture, ×200. In contrast to the growth of HT-29 in monoculture (*B*), when co-cultivated with normal colon fibroblasts, HT-29 forms polypoid structures (*C*) which are similar to in vivo polyps. *Arrows* at the base of the polyp indicate fibroblasts. In contrast to the monocultures of HT-29KM (*D*), HT-29KM in co-culture grows over beads and differentiates forming signet ring cells (*small triangles*), glandular structures, and cellular organization (*E*).

of these associations, thus exhibiting a truly cooperative relationship (16,23).

Production of specific extracellular matrix components found in the developing rat intestine has been directly associated with the differentiation and maturation of these tissues (41). Simon-Assmann et al. (41) have shown that from 14 days of gestation through adulthood, laminin, nidogen, fibronectin, and type IV collagen were present as well as type III procollagen, with fibronectin and type III procollagen disappearing during villius outgrowth. In recent years, glycosaminoglycans (GAGs) have also been found to play a major role in these developmental processes. Studies of rat and chick intestinal development have documented the deposition of intestinal heparan sulfate proteoglycan (HSPG) at the epithelial-mesenchymal interface during maturation. Remodeling of this GAG is thought to be essential for the regulation of cell behavior during morphogenesis, with production of HSPG attributed exclusively to the epithelial component (39). Bouziges et al. (4) state that in addition to the down regulation of heparin sulfate and hyaluronic acid, chondroitin sulfate synthesis was modified to an alternative form as maturation of the tissues progressed. Interestingly, HT-29 human colonic adenocarcinoma studies have been conducted that delineate the production of these molecules in undifferentiated vs. differentiated cells. Undifferentiated HT-29 cells reflect synthesis and secretion of hyaluronic acid and heparan sulfate and one class of chondroitin sulfate.

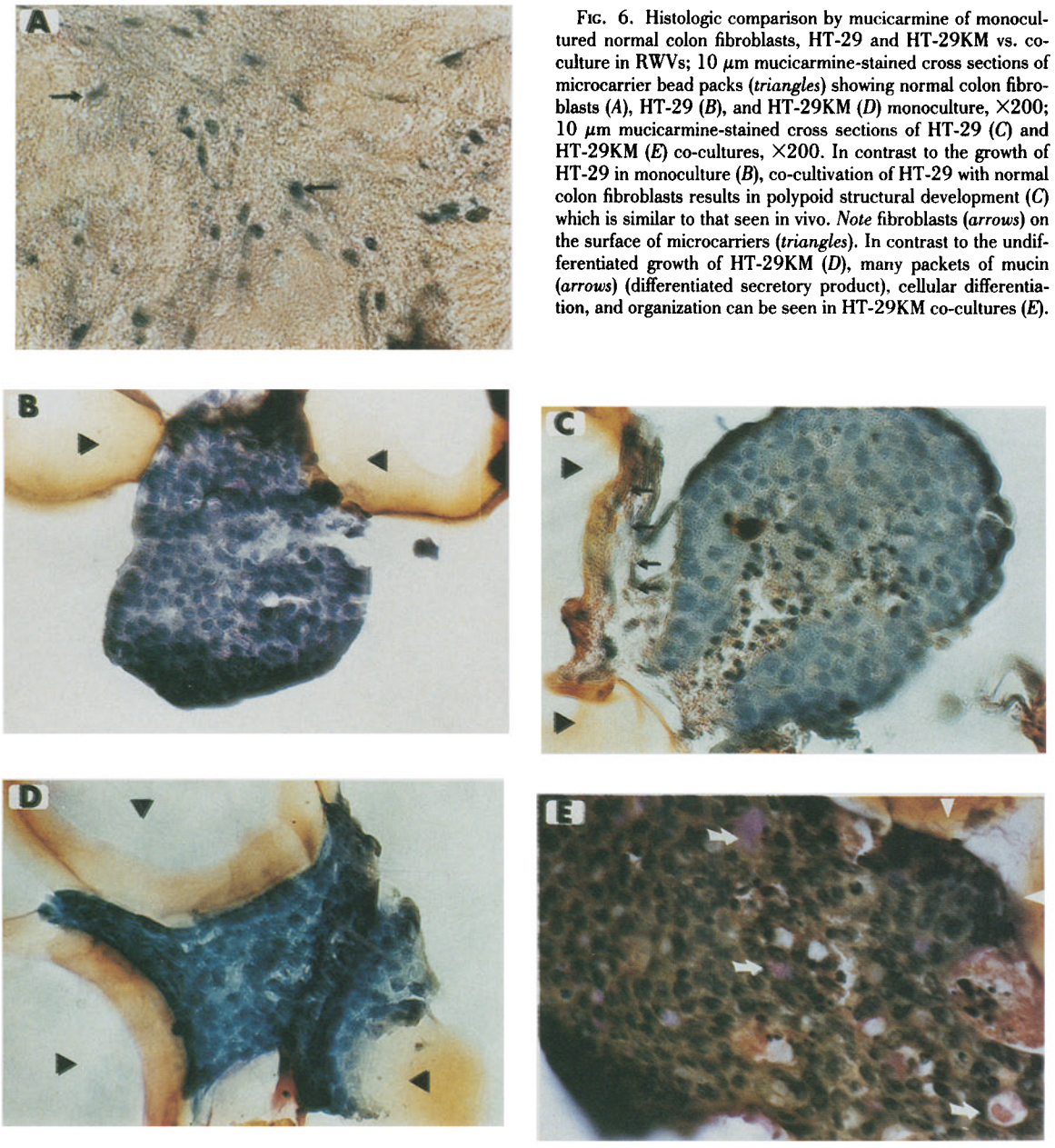


FIG. 6. Histologic comparison by mucicarmine of monocultured normal colon fibroblasts, HT-29 and HT-29KM vs. co-culture in RWVs; 10 μm mucicarmine-stained cross sections of microcarrier bead packs (*triangles*) showing normal colon fibroblasts (*A*), HT-29 (*B*), and HT-29KM (*D*) monoculture, ×200; 10 μm mucicarmine-stained cross sections of HT-29 (*C*) and HT-29KM (*E*) co-cultures, ×200. In contrast to the growth of HT-29 in monoculture (*B*), co-cultivation of HT-29 with normal colon fibroblasts results in polypoid structural development (*C*) which is similar to that seen in vivo. *Note* fibroblasts (*arrows*) on the surface of microcarriers (*triangles*). In contrast to the undifferentiated growth of HT-29KM (*D*), many packets of mucin (*arrows*) (differentiated secretory product), cellular differentiation, and organization can be seen in HT-29KM co-cultures (*E*).

Differentiation of HT-29 in the absence of glucose led to the production of an additional class of chondroitin sulfate (CS4) concomitant with a decrease in heparan sulfate that was no longer secreted into the culture medium (38).

In these studies we report the induction of differentiation in two pluripotent human adenocarcinoma cell lines, HT-29 and HT-29KM, without constitutive change in the growth medium. The inductive and cooperative effects between mesenchyma and epithelia documented by Kédinger et al. (21–23) seem to be evident in this new model system. A primary contrast would be the capability of normal adult colonic fibroblasts to elicit differentiation of, as well as receive the appropriate signals from, neoplastic colonic epithelium to form complex differentiated tissues.

Initial cytohistochemical studies of this model system suggest the production of laminin, type IV collagen, and fibronectin (Fig. 10 *D,E*) as well as other extracellular matrices. Analyses for the presence of GAG molecules discussed above and their role in the development of this model are the subject of current investigations.

The RWV's function is based on two important design principles: a) solid-body rotation, and b) oxygenation by silicone rubber membrane. Solid-body rotation is accomplished by horizontally rotating a vessel (e.g., a cylinder) which is completely filled with culture media (no gas-liquid interface). As the vessel rotates, the liquid inside accelerates, due to viscous coupling, until the entire fluid mass is rotating at the same angular rate as the wall. This environment eliminates the boundary layer inherent in bioreactors in which

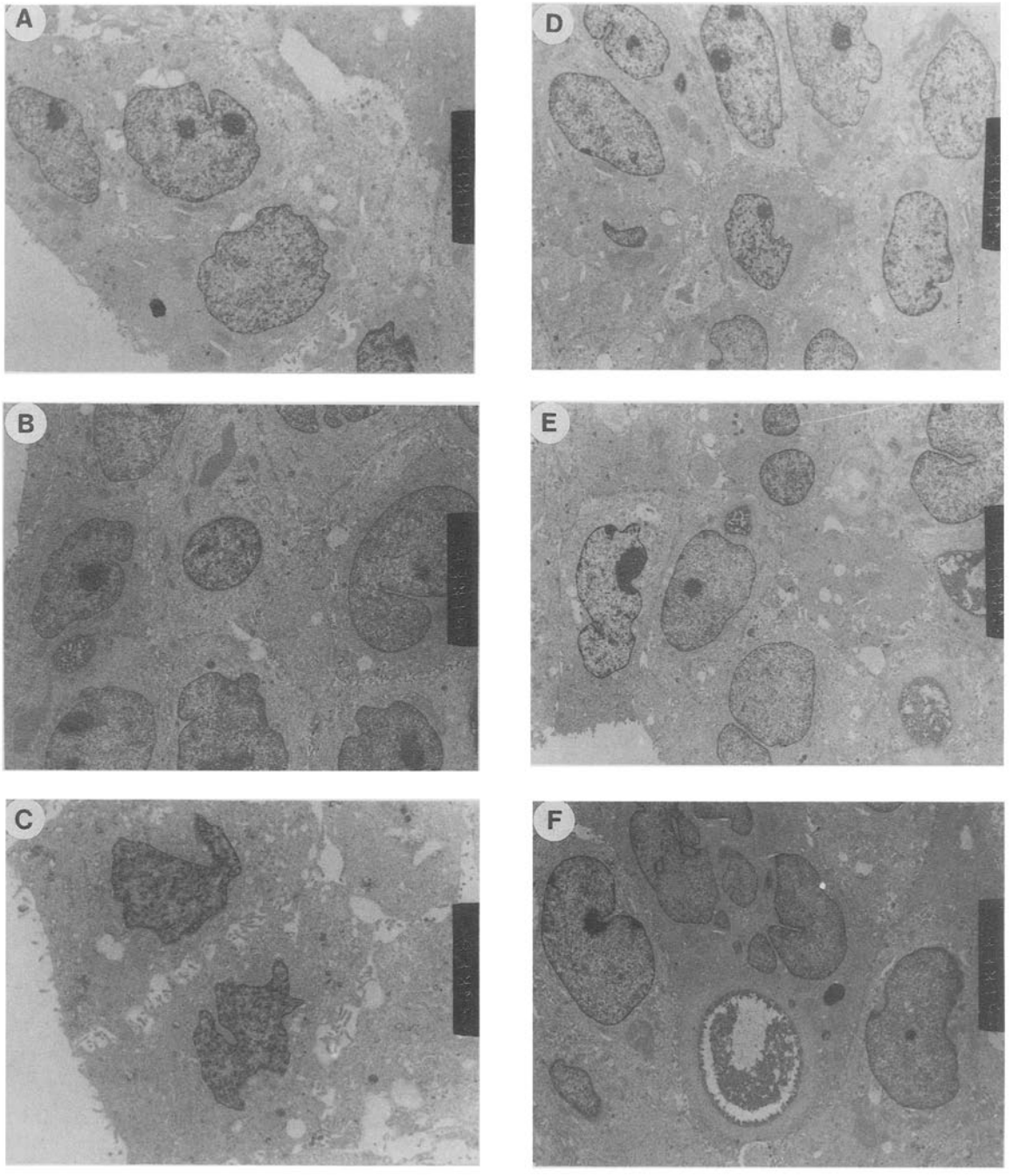


FIG. 7. Comparison TEMs of HT-29 and HT-29KM in RWV monoculture, ×10 300 (*A,B*) and ×17 000 (*C*). *Note* the multiple nuclei and nucleoli (*A,B*), poor cellular junctions (*A,C*), and overall aberrant cell morphology. HT-29KM grown in monoculture on microcarriers in RWVs, ×8100 (*D,E*) and ×10 300 (*F*). *Note* the irregularity of the nuclei and nucleoli and large open spaces at the cell-cell junctions (*D,E*). Characteristic intraglandular structure occasionally found in HT-29KM *F*.

the medium moves relative to the vessel wall. Microcarriers and cells in this environment obey simple kinematics and are uniformly suspended in the fluid provided the rotational speed is correct (35).

This horizontally rotating culture vessel simulates some aspects of microgravity, reducing to a minimum shear and turbulence normally associated with impeller-driven, stirred bioreactors. Designed with no internal moving parts, the vessel described here operates in an unusually low shear regimen. Mixing and shear forces in this culture vessel are due to the microcarrier motion in the media and the contact that the microcarriers occasionally make with the wall and each other. These forces are minimal compared to shear forces in a stirred bioreactor system (35).

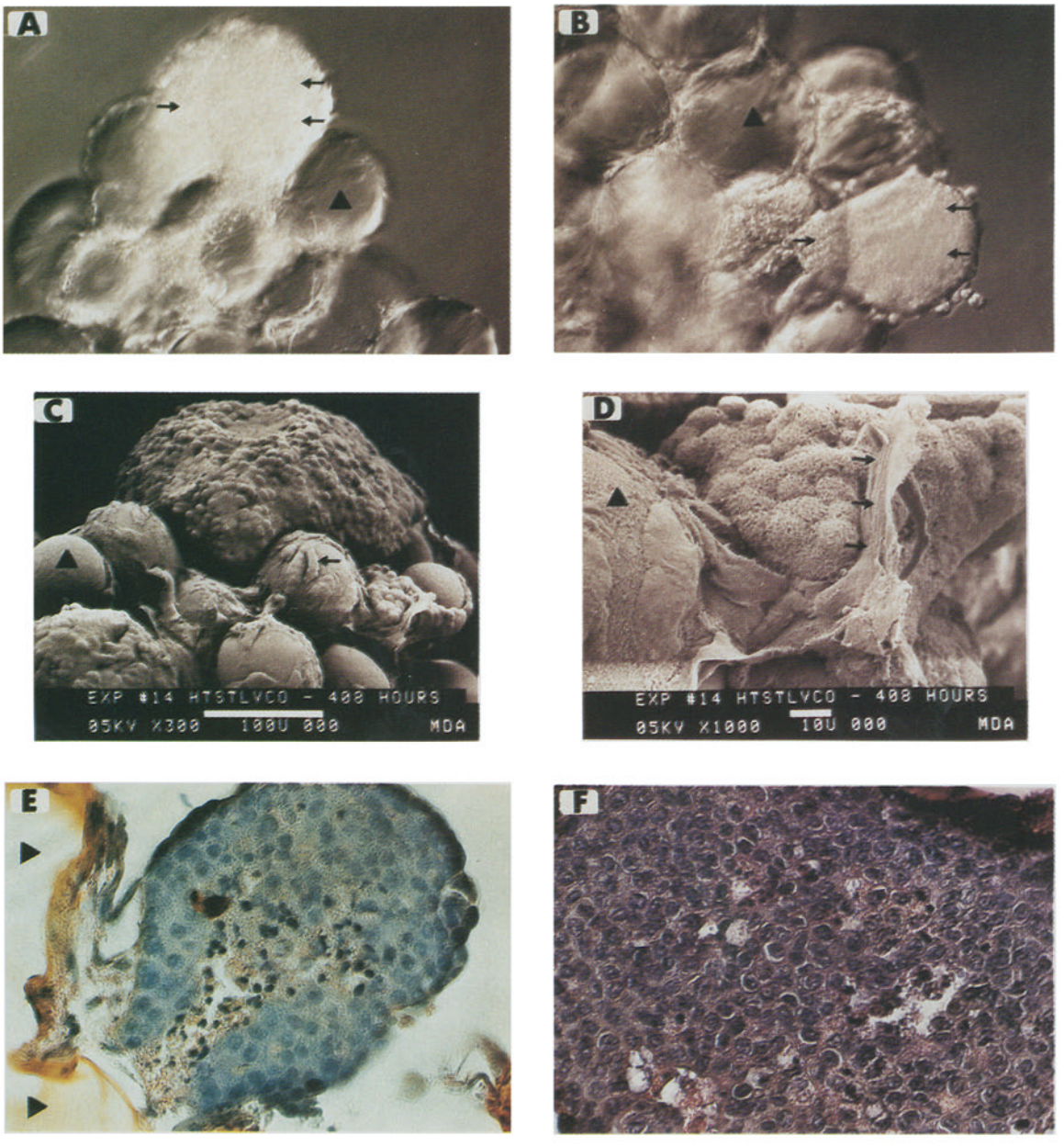


FIG. 8. Co-cultures of HT-29 in RWVs analyzed by light microscopy, SEMs, and histologic staining. *A,B*, light microscopy of HT-29 co-cultured with normal colon fibroblasts (*note* polyps, *arrows*), ×200. SEMs of HT-29 co-cultured on microcarriers, ×300 (*C*) and ×1000 (*D*). *C*, normal colon fibroblasts are seen growing on microcarriers with a large tumor polypoid atop the bead pack. *D*, SEM ×1000 of *C* showing the cell-to-cell interaction between fibroblasts (*arrows*) and tumor cells; 10μm mucicarmine-stained cross sections of HT-29 co-cultivated on microcarriers (triangles), ×200 (*E*) and ×400 (*F*). *E*, polypoid (*arrows*) with center invasion of normal colon fibroblasts similar to in vivo tumor polyps.

The ability of adult colonic mesenchymal cells to function in a manner similar to fetal mesenchyme may be in part based on the quiescent nature of this culture system. Fetal mesenchyme undergoes significant adaptation and change during the transition period from prenatal to postnatal development (23). The adaptive nature may account for the ability of fetal mesenchyme to grow in standard culture systems where adult fibroblasts exhibit a tendency to undergo senescence. The fluid dynamics of the RWV are extremely quiescent, with very low turbulence and shear. This effect can be compared to that of a microcarrier being allowed to fall in a long column of fluid, never reaching the bottom. Particles (e.g., cells and microcarriers) moving through the fluid stay near each other for long periods, allowing cells to construct bridges between microcarriers. Because the vessel has no agitator or mixing device, these

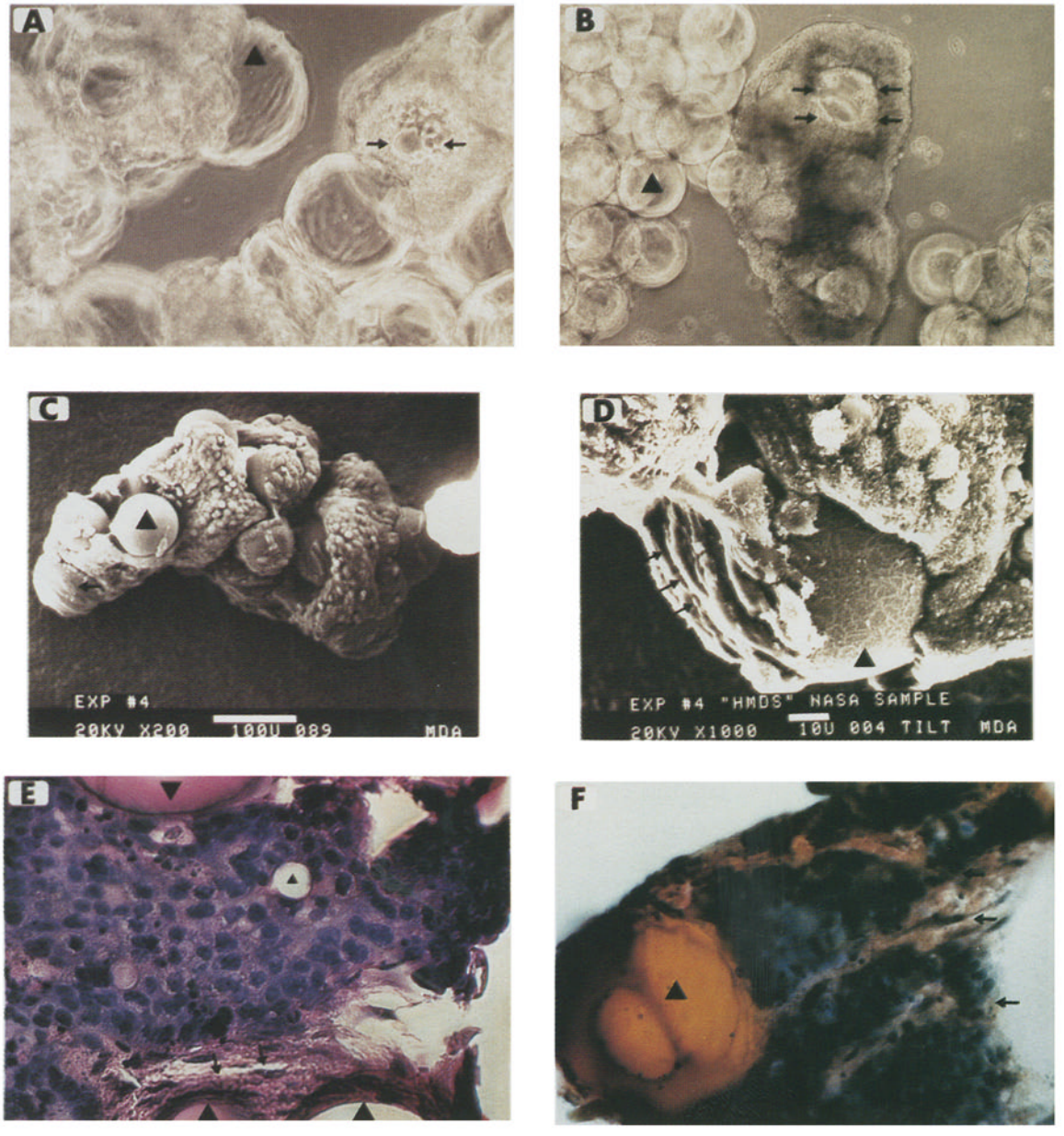


FIG. 9. Co-cultures of HT-29KM in RWVs analyzed by light microscopy, SEMs, and histologic staining. Light microscopy of HT-29KM co-cultivated with normal colon fibroblasts, ×200 (*A*) and ×100 (*B*). *A*, microcarrier (*triangle*) covered with fibroblasts; pseudo gland (*arrows*) which has formed from cells. *B*, a large differentiating mass of HT-29KM tumor cells; large pseudo gland (*arrows*). *C,D*, SEMs of HT-29KM co-cultivated on microcarriers with normal colon fibroblasts. *D*, closeup of a microcarrier with elongated fibroblasts and an over layer of HT-29KM tumor cells. *E,F*, 10 μm H&E- and mucicarmine-stained cross section of HT-29KM co-culture ×200. *Note* the signet ring cell formation in the upper right (*small triangle*). *F*, two microcarriers (*large triangle*) are visible and fibroblasts (*arrows*) can be seen growing across the surface. Microcarrier (*triangle*) from which sinusoids radiate (*arrows*).

bridges are not destroyed by fluid or mechanical shear. In the RWV we have found that three-dimensional aggregates incorporate dozens of microcarriers and replicate experiments have consistently produced large masses of viable cells. The complex aggregation of masses of cells and microcarriers in this culture system is similar to some aspects of tissue formation. Recent data suggest that extracellular matrix and basal lamina formation play a significant role in the three-dimensional structure of the tissues formed in the RWV. Experiment results indicate that microcarrier co-cultures in RWVs may prove useful for studies that require large amounts of high-density, well-organized cellular material (18,35).

The importance of a successful, large-scale, three-dimensional in vitro culture system for intestinal epithelial cells cannot be overemphasized. First, if large, differentiated masses of epithelial tissues

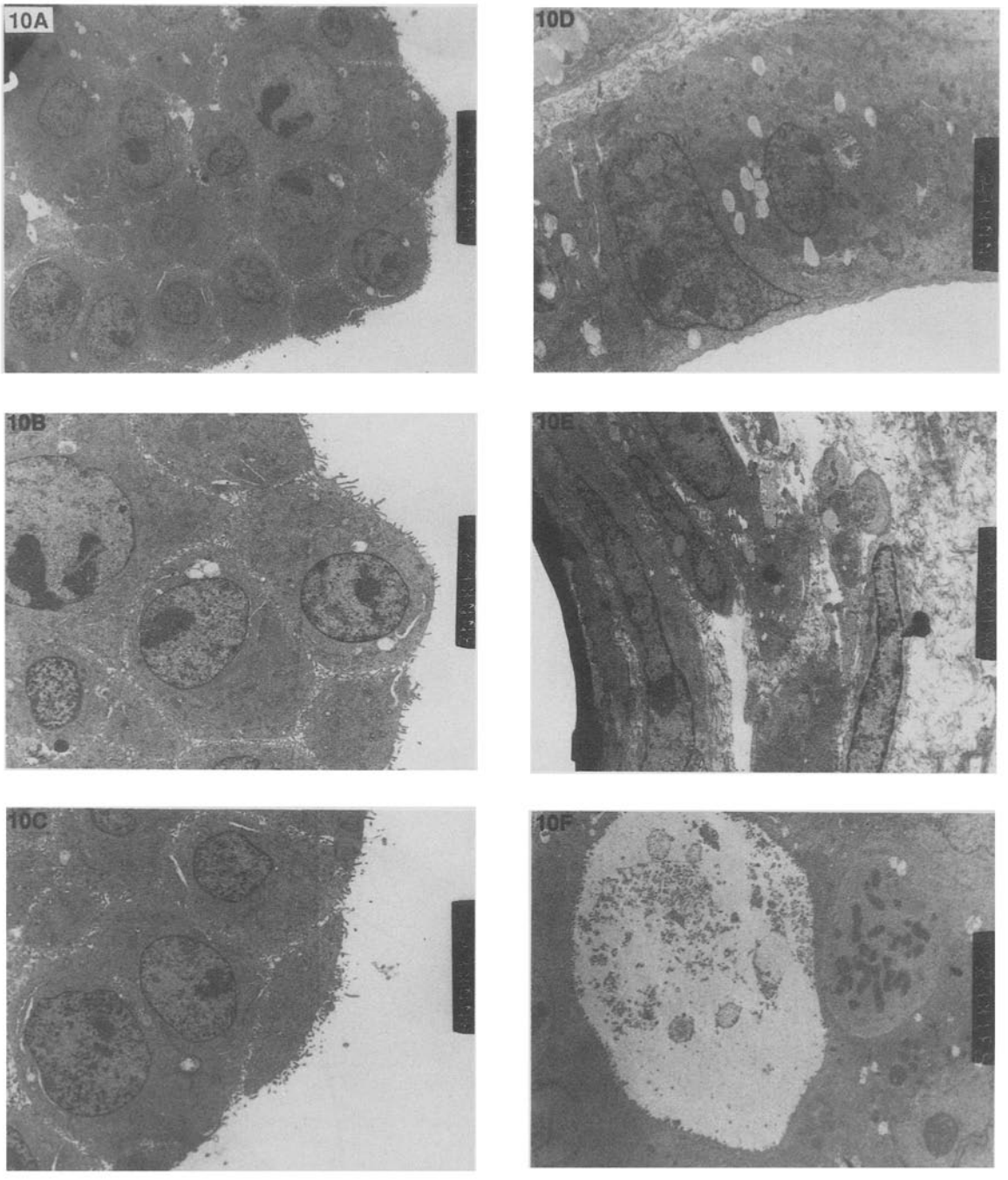


FIG. 10. TEMs of HT-29KM in RWV co-culture. HT-29KM co-cultivated with normal colon fibroblasts, ×5400 (*A*) and ×9000 (*B,C*). *Note* the regularity of the nuclei and reduction in multiple nucleoli as compared to Fig. 6 *D* and *E*. *Note* the presence of an apical microvillus border (*A,–C*) and intercellular gland structures (*F*). HT-29KM co-cultured with normal colon fibroblasts, ×15 000 (*D*), ×10 300 (*E*), and ×7000 (*F*). *Note* the columnar epithelium, tight cellular junctions, and sinusoid formation in *D*. Normal colon fibroblasts layered over a microcarrier and large amounts of extracellular matrix produced in co-culture (*E*). *F*, interglandular structure formed by the junction of several cell borders with internal microvilli.

grow in RWVs, then it ultimately may be possible to use these vessels to propagate even more complex tissues, possibly organs. The RWV strategy offers obvious advantages because the use of autologous tissue minimizes chances of rejection. Second, the availability of large masses of differentiated tissues may provide the opportunity to harvest growth and differentiation factors in higher yield than achieved in standard bioreactors and thus facilitate their isolation and study. Such factors are not isolated easily from tissues

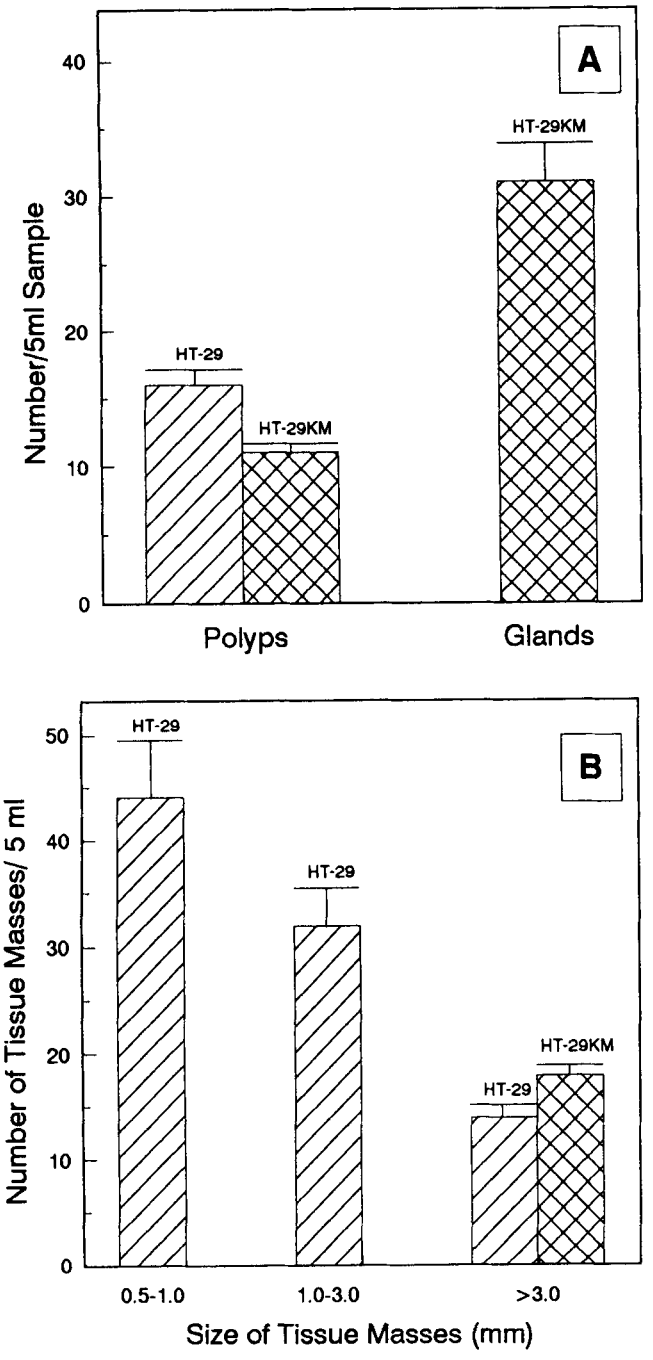


FIG. 11. Five-milliliter samples were harvested from co-cultures in the RWV at 15 to 19 days post-initiation. Samples were plated in 35-mm petri dishes that contained a 2-mm grid. The number of polypoid and gland structures were quantitated against the grid in the dish (*A*). Samples described in (*A*) were measured on a 2-mm gridded petri dish to determine the relative size of the tissue masses (*B*). Tissue masses larger than 0.5 cm were not measured in petri dishes but with a centimeter ruler due to the size of the extraction port.

in vivo because the presence of diverse type of cells confounds isolation attempts. Through improved control of the mesenchymal elements it is conceivable to isolate growth factors at higher concentration. Further, if induction signals are not secreted but rather transmitted from cell-to-cell (10), the availability of larger tissues in culture will facilitate identification of intermediary gene products and peptides under controlled conditions. Thus, this new model system has important implications for studies in organogenesis as well as oncology.

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REFERENCES

1. Blay, J.; Brown, K. D. Functional receptors for epidermal growth factor in an epithelial-cell line derived from the rat small intestine. Biochem. J. 225:85–94; 1985.
2. Blay, J.; Brown, K. D. Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. J. Cell. Physiol. 124:107–112; 1985.
3. Blay, J.; Brown, K. D. Contradistinctive growth responses of cultured rat intestinal epithelial cells to epidermal growth factor depending on cell population density. J. Cell. Physiol. 129:343–346; 1986.
4. Bouziges, F.; Simon-Assmann, P.; Simo, P., et al. Changes in glycosaminoglycan expression in the rat developing intestine. Cell Biol. Int. Rep. 15:2:97–106; 1991.
5. Brattain, M. G.; Kimball, P. M.; Pretlow, T. G. 2d, et al. Partial purification of human colonic carcinoma cells by sedimentation. Br. J. Cancer 35:850–857; 1977.
6. Buset, M.; Winawer, S.; Friedman, E. Defining conditions to promote the attachment of adult human colonic epithelial cells. In Vitro Cell. Dev. Biol. 23:403–412; 1987.
7. Chantret, I.; Barbat, A.; Dussaulx, E., et al. Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines. Cancer Res. 48:1936–1942; 1988.
8. Corps, A. N.; Brown, K. D. Stimulation of intestinal epithelial cell proliferation in culture by growth factors in human and ruminant mammary secretions. J. Endocrinol. 113:285–290; 1987.
9. Daneker, G. W., Jr.; Mercurio, A. M.; Guerra, L., et al. Laminin expression in colorectal carcinomas varying in degree of differentiation. Arch. Surg. 122:1470–1474; 1987.
10. Durban, E. Mouse submandibular salivary epithelial cell growth and differentiation in long term culture: influence of the extracellular matrix. In Vitro Cell. Dev. Biol. 26:33–43; 1990.
11. El-Deriny, S. E.; O'Brien, M. J.; Christensen, T. G., et al. Ultrastructural differentiation and CEA expression of butyrate-treated human pancreatic carcinoma cells. Pancreas 2:25–33; 1987.
12. Fatini, J.; Galons, J-P.; Marvaldi, J., et al. Growth of human colonic adenocarcinoma cell line (HT 29) on microcarrier beads: metabolic studies by phosphorus nuclear magnetic resonance spectroscopy. Int. J. Cancer 39:255–260; 1987.
13. Fogh, J.; Trempe, G. In: Fogh, J., ed. Human tumor cells in vitro. New York: Plenum Press; 1975:115–159.
14. Fukamachi, H.; Mizuno, T.; Kim, Y. S. Morphogenesis of human colon cancer cells with fetal rat mesenchymes in organ culture. Experientia 42 Birkhäuser Verlag, CH-4010 Basel/Switzerland; 1986.
15. Haake, A. R.; Lane, H. T. Retention of differentiated characteristics in human fetal keratinocytes. In Vitro Cell. Dev. Biol. 25:592–600; 1989.
16. Haffen, K.; Kédinger, M.; Simon-Assmann, P. Mesenchyme-dependent differentiation of epithelial progenitor cells in the gut. J. Pediatr. Gastroenterol. Nutr. 6:14–23; 1987.

17. Haffen, K.; Lacroix, B.; Kédinger, M., et al. Inductive properties of fibroblastic cell cultures derived from rat intestinal mucosa on epithelial differentiation. Differentiation 23:226–233; 1983.
18. Goodwin, T. J.; Jessup, J. M.; Sams, C., et al. In vitro three-dimensional tissue modeling. JSC Technology Annual Report. NASA Technical Memorandum 100473, Washington, DC.
19. Jessup, J. M.; Giavazzi, R.; Campbell, D., et al. Growth potential of human colorectal carcinomas in nude mice: association with the preoperative serum concentration of carcinoembryonic antigen in patients. Cancer Res. 48:1689–1692; 1988.
20. Kaye, G. I.; Pascal, R. R.; Lane, N. The colonic pericryptal fibroblast sheath: replication, migration, and cytodifferentiation of a mesenchymal cell system in adult tissue. 3. Replication and differentiation in human hyperplastic and adenomatous polyps. Gastroenterology 60:515–536; 1971.
21. Kédinger, M.; Simon, P. M.; Grenier, J. F., et al. Role of epithelial-mesenchymal interactions in the ontogenesis of intestinal brush-border enzymes. Dev. Biol. 86:339–347; 1981.
22. Kédinger, M.; Simon-Assmann, P.; Alexandre, E., et al. Importance of a fibroblastic support for in vitro differentiation of intestinal endodermal cells and for their response to glucocorticoids. Cell Differ. 20:171–182; 1987.
23. Kédinger, M.; Simon-Assmann, P.; Lacroix, B., et al. Fetal gut mesenchyme induces differentiation of cultured intestinal endodermal and crypt cells. Dev. Biol. 113:474–483; 1986.
24. Kleinman, D.; Sharon, Y.; Sarov, I., et al. Human endometrium in cell culture: a new method for culturing human endometrium as separate epithelial and stromal components. Arch. Gynecol. Obstet. 234:103–112; 1983.
25. Luna, L. G., editor. Histologic staining methods. Armed Forces Institute of Pathology. American Registry of Pathology, 3rd. ed. New York, London; 1968.
26. Moyer, M. P.; Dixon, P. S.; Culpepper, A. L., et al. In: Moyer, M. P.; Poste, G. H., eds. Colon cancer cells. New York: Academic Press; 1990:85–136.
27. O'Loughlin, E. V.; Chung, M.; Hollenberg, M., et al. Effect of epidermal growth factor on ontogeny of the gastrointestinal tract. Am. J. Physiol. 249:674–678; 1985.
28. Phillips, T. E.; Huet, C.; Bilbo, P. R., et al. Human intestinal goblet cells in monolayer culture: characterization of a mucus-secreting subclone derived from the HT29 colon adenocarcinoma cell line. Gastroenterology 94:1390–1403; 1988.
29. Pinto, M.; Appay, M-D.; Simon-Assmann, P., et al. Enterocytic differentiation of cultured human colon cancer cells by replacement of glucose by galactose in the medium. Biol. Cell. 44:193–196; 1982.
30. Podolsky, D. K.; Fournier, D. A.; Lynch, K. E. Human colonic goblet cells. Demonstration of distinct subpopulations defined by mucin-specific monoclonal antibodies. J. Clin. Invest. 77:1263–1271; 1986.
31. Polak-Charcon, S.; Hekmati, M.; Ben-Shaul, Y. The effect of modifying the culture medium on cell polarity in a human colon carcinoma cell line. Cell Diff. Dev. (Ireland) 26:119–129; 1989.
32. Pyke, K. W.; Gogerly, R. L. Murine fetal colon in vitro; assays for growth factors. Differentiation 29:56–92; 1985.
33. Reid, L. M.; Jefferson, D. M. Culturing hepatocytes and other differentiated cells. Hepatology 4:548–559; 1987.
34. Richman, P. I.; Bodmer, W. F. Control of differentiation in human colorectal carcinoma cell lines: epithelial-mesenchymal interactions. J. Pathol. 156:197–211; 1988.
35. Schwarz, R. P.; Goodwin, T. J.; Wolf, D. A. Cell culture for three-dimensional modeling in rotating-wall vessels: applications of simulated microgravity. J. Tissue Cult. Methods 13:4; 1991.
36. Shamsuddin, A. In: Moyer, M. P.; Poste, G. H., eds. Colon cancer cells. New York: Academic Press; 1990:137–153.
37. Sheehan, D. C.; Hrapchak, B. B., editors. Theory and practice of histotechnology, 2nd ed. St. Louis, MO: C. V. Mosby Co.; 1980.
38. Simon-Assmann, P.; Bouziges, F.; Daviaud, D., et al. Synthesis of glycosaminoglycans by undifferentiated and differentiated HT29 human colonic cancer cells. Cancer Res. 47:4478–4484; 1987.
39. Simon-Assmann, P.; Bouziges, F.; Vigny, M., et al. Origin and deposition of basement membrane heparan sulfate proteoglycan in the developing intestine. J. Cell Biol. 109:1837–1846; 1989.
40. Simon-Assmann, P.; Kédinger, M.; Grenier, J. F., et al. Organ culture of fetal rat intestine. Enzyme 31:65–72; 1984.
41. Simon-Assmann, P.; Kédinger, M.; Haffen, K. Immunocytochemical localization of extracellular-matrix proteins in relation to rat intestinal morphogenesis. Differentiation 32:59–66; 1986.
42. Sutherland, R. M. Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 240:177–184; 1988.
43. Viallard, V.; Denis, C.; Trocheris, V., et al. Effect of glutamine deprivation and glutamate or ammonium chloride addition on growth rate, metabolism and differentiation of human colon cancer cell-line HT29. Int. J. Biochem. 18:263–269; 1986.
44. Wiens, D.; Park, C. S.; Stockdale, F. E. Milk protein expression and ductal morphogenesis in the mammary gland in vitro: hormone-dependent and -independent phases of adipocyte-mammary epithelial cell interaction. Dev. Biol. 120:245–258; 1987.
45. Wolf, D. A.; Schwarz, R. P.; Sams, C. F. Bioreactor incubator tissue culture system. Johnson Space Center Research and Technology Report. Annual Report 33–34; 1989.