Rotating-Wall Vessel Coculture of Small Intestine as a Prelude to Tissue Modeling: Aspects of Simulated Microgravity (43525)

THOMAS J. GOODWIN,*[,1] WILLIAM F. SCHROEDER,[†] DAVID A. WOLF,[‡] AND MARY PAT MOYER[†]

KRUG Life Sciences, Houston, Texas 77058; University of Texas Health Science Center at San Antonio,[†] San Antonio, Texas 78284; and NASA, Johnson Space Center,[‡] Medical Operations and Research Branch, Houston, Texas 77058*

Abstract. **A new low shear stress, low turbulence microcarrier culture system has been developed at NASA's Johnson Space Center that permits large-scale three-dimensional tissue culture. Tissue culture bioreactors called rotating-wall vessels were used in conjunction with multicellular cocultivation to develop a unique *in vitro* tissue-modeling system. Normal small intestine epithelium and mesenchymal cells were cocultivated on Cytodex-3 microcarriers and were initiated in two phases. Normal small intestine mesenchymal cells were inoculated into the rotating-wall vessel at 2×10^5 cells/ml and allowed to attach and proliferate for 2 to 3 days. Normal small intestine epithelium was then added at an innoculum of 2×10^5 cells/ml and cultivation continued for 30 to 40 days. These cocultures attained cell numbers of $4\text{–}6 \times 10^6$ cells/ml and differentiated to form tissue-like masses of 0.4–0.5 cm with minimal necrosis. The masses displayed apical brush borders, differentiated epithelial cells, cellular polarity, extracellular matrix, and basal lamina. Verification of mesenchymal and epithelial cell expression was determined by immunocytochemistry and scanning electron microscopy. These data suggest that the rotating-wall vessel affords a new tissue culture model for investigation of growth, regulatory, and differentiation processes within normal tissues.**

[P.S.E.B.M. 1993, Vol 202]

Cellular differentiation involves complex cellular interactions (1–3) in which cell membrane junctions, extracellular matrices (e.g., basement membrane and ground substances), and soluble signals (endocrine, autocrine, and paracrine) play an important role (4–7). This process is also influenced by the spatial relationships of cells to each other, with responsiveness to exogenous mediators requiring more than simply the presence of receptors. This is exemplified by isolated endometrial epithelium lacking estrogen response despite the presence of receptors (8) and the improved culture longevity, retention of ultrastructural markers, and tissue-specific functions, such as hydrocortisone-inducible tyrosine aminotransferase (9, 10), in hepatocytes cocultured with fibroblasts, Kupffer cells, and/or endothelial-feeder layers. The need for complex cell interactions is further supported by the inability to use conditioned medium from a "feeder" cell line to eliminate the need for cocultivation (9–12).

Culture of normal or neoplastic small intestine epithelium as large-scale, three-dimensional configurations is not possible with routinely used *in vitro* culture technology (13). Thus, factors that control proliferation and differentiation in the gastrointestinal tract remain largely unknown (14–19). Short-term cultures have been accomplished by a variety of methods for either animal or human cells (1, 4, 8, 9). However, long-term growth has required complex, defined culture media (20) or *in vitro* transformation to increase longevity (21–23). Organ culture permits only short-term analysis of parental population dynamics. Long-term cultures (longer than 7 weeks) (23) have been difficult to achieve, since crypt cells are unable to survive standard culture regimens, and two-dimensional organ cultures do not support the *de novo* assembly of stroma and its contribution to epithelial cell growth. In the absence of a successful and consistently reproducible long-term methodology to culture gut epithelium, a new technol-

[1] To whom requests for reprints should be addressed at KRUG Life Sciences, 1290 Hercules, Suite 120, Houston, TX 77058.

Received June 11, 1991. [P.S.E.B.M. 1993, Vol 202]
Accepted July 20, 1992.

0037-9727/93/2022-0181$3.00/0

Table I. GTSF-2 Formulation

Component	Concentration	Source/order no. of designation
MEM-α supplemented with 2.25 g/liter $NaHCO_3$	400 ml (40%)	Gibco/430-1900EB
L-15	600 ml (60%)	Gibco/430-1300EB
$NaHCO_3$	1.35 g/liter	Sigma/S-5761
HEPES	3.0 g/liter	Research Organics/6003H-2
Folic acid	6.667 μg/liter	Sigma/F-8758
0.5% Nicotinic acid	0.667 ml/liter	Sigma/N-4126
Bactopeptone	0.6 g/liter	Difco/0118-01
i-Inositol	0.024 g/liter	Sigma/I-5125
Fructose	0.13 g/liter	Sigma/F-3510
Galactose	0.25 g/liter	Sigma/G-5388
D-Glucose	1.0 g/liter	Sigma/G-5250
300 m*M* L-Glutamine	10 ml/liter	Sigma/G-5763
Gentamycin	1 ml/liter	Gibco/600-5750AD
Fungizone	2 ml/liter	Gibco/600-5295AE
Insulin-transferrin-sodium-selenite	5 ml/liter	Sigma/I-1884
Fetal bovine serum	60 ml (6%)	Hyclone/A-1111-L
pH adjusted to 7.4 with 1 *N* NaOH		

ogy developed at NASA's Johnson Space Center is now being used to construct a large-scale, three-dimensional *in vitro* tissue culture model (24, 25). This technology involves horizontally rotating cylindrical tissue culture vessels, or rotating-wall vessels (RWV) (26), that provide controlled supplies of oxygen and nutrients, with minimal turbulence and extremely low shear (27). These vessels rotate the wall and the fluid inside at the same speed, thus continuously randomizing the gravity vector and holding particles (i.e., microcarriers and cells) relatively motionless in the fluid, thereby simulating some aspects of microgravity (27, 28). When used in concert with microcarriers and cocultivation of epithelial and mesenchymal cells, the RWV allow the three-dimensional assembly of cells into tissue-like masses. In this study, we report our achievements with small intestine epithelial cells.

Materials and Methods

Source and Culture of Cells Used for Seeding. Human small intestine epithelial and mesenchymal cells were obtained from normal organs of patients, predominantly organ transplant donors, with no evidence of disease. All were harvested and banked at the University of Texas Health Science Center at San Antonio (Center for Human Cell Biotechnology). Cells were initiated in cultures and propagated in media supplemented with 2% fetal bovine serum (FBS) designated M3:2 for epithelial cells or with 10% FBS (M3:10) for mesenchymal cells, as described elsewhere (21, 22). M3 medium, supplied through services of the Center for Human Cell Biotechnology, is a complex base medium supplemented with many growth factors and nutrients (21, 29). All cell cultures were grown in a Forma humidified CO_2 incubator with 95% air and 5% CO_2, and constant atmosphere at a temperature of 37°C. Normal mesenchymal cells were passaged as required by enzymatic dissociation with a solution of 0.1% trypsin and 0.1% EDTA for 15 min at 37°C. 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-flasks with 25 ml of fresh growth medium. Small intestine epithelial cells were passaged as required by dilution at a 1:2 ratio into fresh M3:2 medium in T-flasks. Cultures of epithelial cells were pooled cultures derived from the ileum and jejunum of three male donors (22–35 years of age). Mesenchymal cells were obtained from the submucosa of five donors (four males, 17–27 years old, and a 2-month-old female). Viable cocultures grown in the RWV were harvested over periods up to 41 days and prepared for various assays or cultured in M3:2 (to promote epithelial cell outgrowth) and M3:10 (to promote fibroblast outgrowth).

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 cocultivation 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.

RWV Cultures. The RWV is a horizontally rotated, zero headspace, center oxygenation, transparent culture vessel. Cells to be cultured in the RWV were initially grown in T-flasks, as described above, in preparation for seeding into the vessels. Normal mesenchymal cells were removed from T-75 flasks by enzymatic digestion, washed once with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), and assayed for viability by trypan blue dye exclusion (Gibco). Cells were held on ice in fresh growth medium until inoculation. The primary inoculum for each coculture experiment was 2×10^5 mesenchymal cells/ml in a 125-ml vol with 5 mg/ml of Cytodex-3 microcarrier beads (Pharmacia, Piscataway, NJ). Cytodex-3 microcarriers were Type I, collagen-coated dextran beads, 175 microns in diameter. Cultures were allowed to grow for a minimum of 2–3 days before the medium was changed and 2×10^5 epithelial cells/ml were added. Thereafter, fresh medium was replenished by 65% of the total vessel volume each 20–24 hr. As metabolic requirements increased, fresh medium was supplemented with an additional 100 mg/dl of glucose.

Medium for Coculture Experiments. Initial coculture experiments were performed using a 1:1 mixture of M3:2 medium and standard minimal essential medium-α (MEM-α; Gibco) supplemented with 10% FBS. As the culture conditions were refined, a medium formulated at NASA's Johnson Space Center, GTSF-2, detailed in Table I (patent pending), was found to meet the growth requirements of the coculture system without the need for unique growth factors and most of the other complex components found in M3:2. GTSF-2 is a trisugar-based medium, modified from GTSF-1, containing glucose, galactose, and fructose supplemented with 6% FBS.

Growth Curves. The cocultures were sampled over the course of the experiments. Due to the slow cell growth and development of the tissue material, sampling began at approximately Day 7 and continued at 4- to 6-day intervals throughout the experiment. The methodology for determination of the total number of cells/ml in the cultures was as follows: the mean number of cells per bead was determined from three replicate 1-ml homogeneously suspended samples taken from the reactor vessels at selected intervals. First, the number of cells was determined essentially by the method described for counting released nuclei (30). For these counts, the 1-ml samples of cell-bead suspension were placed in 15-ml centrifuge tubes and the medium was aspirated. The samples were washed once with CMF-PBS and 1 ml of 0.1% crystal violet/0.1 *M* citric acid staining solution was added. Tubes were placed in the CO_2 incubator for 1 hr. Nuclei were completely released from cells by brief vortex mixing and three 25-μl samples were taken from the uniform suspension to count released nuclei with a hemacytometer.

The number of beads per sample was evaluated by a method developed in our laboratory. After the samples were removed for nuclei counts, the beads remaining in the centrifuge tubes were washed twice with 15 ml of CMF-PBS to remove the staining solution. The CMF-PBS was aspirated except for 1 ml after the last rinse. A 100-ml volumetric flask was filled with Isoton (Curtin Matheson Scientific, Houston, TX) and 10 ml were poured into the tubes containing the beads. The suspended beads were then poured into a 200-ml round-bottomed Coulter counting beaker. The centrifuge tubes were rinsed three more times or until no beads were left in the tube. The remaining Isoton was poured into the counting beaker and beads were counted while stirring on a model ZM Coulter counter calibrated for the 560-μm aperture tube. The following formula was used to determine the average number of cells per bead.

Beads per sample

$$= \frac{\text{1-ml } A + \text{100-ml } B \times C \times \text{bead count}}{\text{2-ml sample volume counted}^2} \quad [1]$$

Cells per sample

$$= \text{nuclei/ml (from hemacytometer count)} \times C \quad [2]$$

$$\text{Cells per bead} = \frac{\text{cells/sample}}{\text{beads/sample}} \quad [3]$$

where *A* is the volume of bead suspension (1 ml), *B* is the diluent (Isoton) in the Coulter counter beaker, and *C* is the original volume of the sample.

Immunocytochemistry. Antibodies used to detect cell-type-specific markers are shown in Table II. The keratin, vimentin, and Factor VIII antibodies detect epithelial, fibroblastic, and endothelial cells, respectively (28, 29, 31–33). Villin is a cytoskeletal protein found only in epithelial cells from small intestine and colon (11). The angioblast marker is present in subsets of precursor endothelial cells, particularly dividing cells. Sucrase is an enzyme found in the epithelial cell brush border of the small intestine (34). Basement membrane and extracellular matrix components (e.g., laminin, fibronectin, Collagen IV, and proteoglycan) were also assayed to determine their expression in the tissue-like structures.

Cell cocultures grown on Cytodex-3 microcarriers in the NASA RWV were fixed in OmniFix, an alcohol-based fixative not containing aldehydes or mercury (Xenetics Biochemic, Tustin, CA). At all times, extreme care was taken not to damage the delicate tissue-like structure composed of cellular material and microcarrier beads. When an abundance of beads was present, the supernatant fluid was carefully decanted and a sample of beads was enclosed in a biopsy bag, then placed in a cassette to prepare a paraffin block. When bead clusters were scarce, a Shandon Cytoblock kit

[2] For bead counts, the ZM model Coulter counter, equipped with the 560-μm aperture tube, was set to draw a 2-ml vol of the Isoton-bead suspension. The suspension was stirred constantly during counting. Each bead sample was counted a minimum of five times, and the means and standard deviations were determined. After completion of the cell/bead count the total number of cells/ml was determined by multiplying the cell/bead number by the number of beads/ml or 2×10^4.

Table II. Antibodies Used in This Study

Antibody specificity	Dilution	Source/order no. or designation
Pancytokeratin	Prediluted	DAKO/L1824
Vimentin	Prediluted	DAKO/L1843
Factor VIII	Prediluted	DAKO/L1809
Villin	1:20	Chemicon/MAB 1671
Sucrase	1:20	A. Elbein, UTHSCSA/YT
Angioblasts	1:20	Accurate Chem/HE3-5/47
Laminin	1:20	ICN/69-630
Fibronectin	1:20	US BIOCH/33752
Proteoglycan	1:20	Biological Products for Science/MCA 326
Collagen Type IV	1:20	DAKO/M785

(Shandon Inc., Pittsburgh, PA) was used. Cassettes were processed in a standard tissue processor. Five-micron sections were cut from the paraffin-embedded tissues, deparaffinized by standard procedures, then assayed by incubation with the test antibodies (Table II), followed by use of the universal labeling streptavidin biotin kit (No. K680; DAKO Inc., Carpenteria, CA), which detects mouse monoclonal and rabbit polyclonal antibodies. All experiments included one positive and two negative controls. The positive control was normal tissue sections or normal cells positive for the primary antibody used. The negative controls were (i) phosphate-buffered saline only, to test for false binding by "link" antibody and streptavidin, and (ii) normal serum of the same species from which the primary antibody was prepared.

For cells from culture, the immunoperoxidase protocols described for the DAKO labeling streptavidin biotin kit were followed with the following exceptions. Cells were cytofuged onto slides or grown as monolayer cultures. For cytofuged suspension cells, slides were soaked in 95% EtOH for 2 min at room temperature in order to remove the carbowax from the collecting fluid. All slides were rinsed in 1× CMF-PBS by dipping six times in a Coplin jar, then immersed in cold acetone for 2 to 3 min at room temperature, and air dried. Where appropriate for the test antibody, a Pronase pretreatment was also done per manufacturer's specifications. All slides were placed in 0.3% H_2O_2 for 10 min at room temperature to remove endogenous peroxides, rinsed in CMF-PBS, then assayed.

Scanning Electron Microscopy. Samples from the RWV cultures were taken for scanning electron microscopy at the same times as those taken for growth curve and immunocytochemistry. After removal from the reactor vessels, samples were washed once with CMF-PBS. The samples were suspended in a buffer containing 3% glutaraldehyde and 2% paraformaldehyde in 0.1 *M* cacodylate buffer at pH 7.4 (35), then rinsed for 5 min with cacodylate buffer three times and postfixed with 1% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, PA) in cacodylate buffer for 1 hr. Samples were then rinsed for 5 min with distilled water three times and then treated for 10 min with a Millipore (Millipore Corp., Bedford, MA) (0.2-μm)-filtered, saturated solution of thiocarbohydrazide (Electron Microscopy Sciences), then washed for 5 min with distilled water five times and fixed with 1% buffered osmium tetroxide for 10 min. This last step was necessary to prevent the microcarriers from collapsing. Samples were then rinsed with distilled water three times and dehydrated with increasing concentrations of EtOH, followed by three changes in absolute methanol. After transfer to 1,1,1,3,3,3-hexamethyldisilazane (Electron Microscopy Sciences), samples were allowed to soak for 10 min, drained, and air dried overnight. Dried samples were sprinkled with a thin layer of silver paint on a specimen stub, dried, coated by vacuum evaporation with platinum-palladium alloy, and then examined in the JEOL T330 scanning electron microscopy at an accelerating voltage of 5 to 10 kV.

Results

Growth Curves. Monocultures of normal primary small intestine epithelium and mesenchymal cells were cultured in T-flasks to determine their maximum cell density and population doubling times. The population doubling time for small intestine epithelia when grown in T-flasks suspension culture was 4–6 days, with a maximum cell density of 10^6 cells/ml (Table III). This required a defined and complex medium (M3:2) composed of multiple, expensive, and/or unique growth factors. Small intestine mesenchymal cells in monolayer culture doubled every 18–24 hr when cultured in M3:10 medium.

Initial coculture experiments in the RWV were grown in 1:1 mixture of M3:2 and MEM-α supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) on 5-mg/ml Cytodex-3 microcarriers (Pharmacia, LKB) (Fig. 1A). Although this medium provided good growth, tissue development was less than optimum. The cultures attained cell densities of 2.3–3.2 × 10^6 cells/ml and although the cultures did not reach plateau phase, suggesting the potential for continued growth, the epithelial cell contingent exhibited signs of considerable stress while the mesenchymal cells proliferated well. In order to balance the nutri-

Table III. Maximum Cell Densities

Culture conditions	Primary	
	Epithelium	Fibroblasts
T-flasks	1.0 × 10^6 [a]	2.0 × 10^6 [b]
Monoculture in RWV on microcarriers	1.0 × 10^6 [a]	0.6 × 10^6 [a]
Coculture in RWV on microcarriers	6.2 × 10^6 [a]	

[a] Data are expressed as cells/ml.
[b] Data are expressed as cells/T-flask.

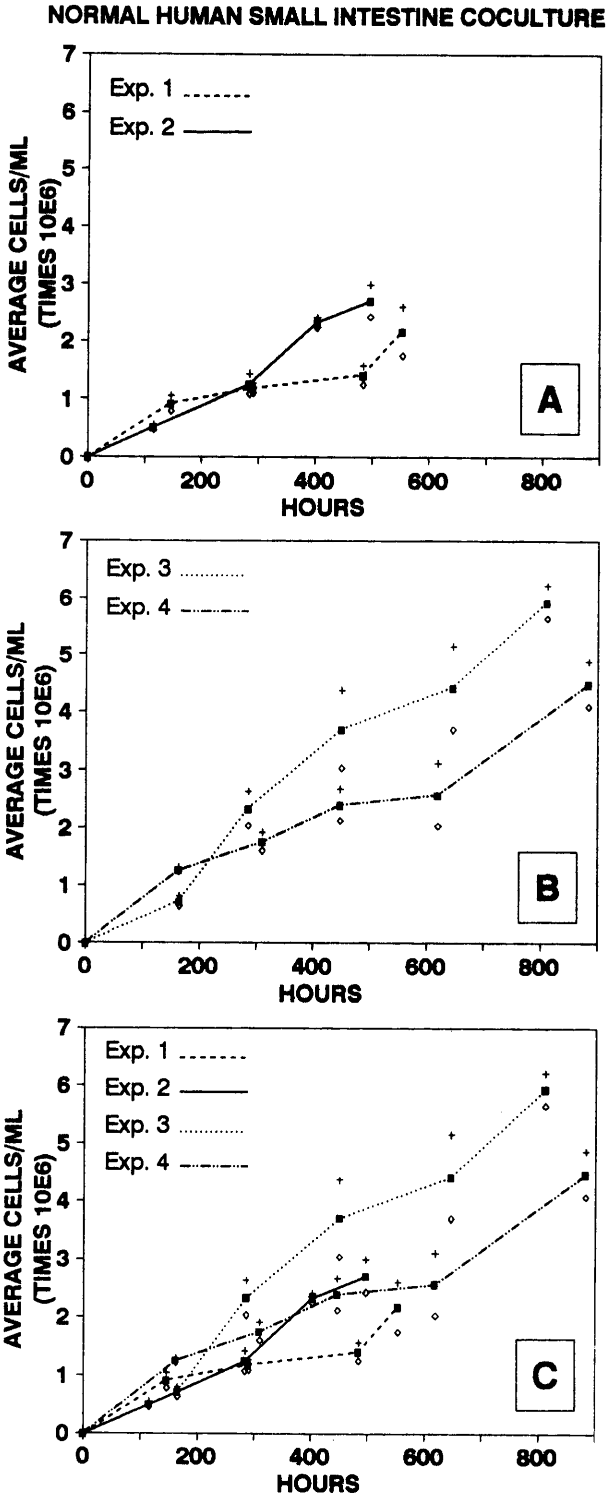

Figure 1. Growth curves of small intestine cocultures. (A) Experiments conducted on a 1:1 mixture of M3:2 and MEM-α growth medium. (B) Experiments conducted on GTSF-2 medium. (C) Comparison of growth curves from experiments performed on a 1:1 mixture of M3:2 and MEM-α and GTSF-2.

tional components for each cell population and decrease the complexity and cost of the culture medium, an alternative medium (GTSF-2) was formulated at Johnson Space Center and found to meet the growth requirements without the need for multiple growth factors and supplements. Figure 1B shows growth curves from two experiments cultured in GTSF-2. These cultures produced slightly higher average cell densities (4.6–6.2 $\times$ 10^6 cells/ml as compared with Figure 1A, which exhibited cell densities of 2.5–3.2 $\times$ 10^6 cells/ml), but more importantly, exhibited an epithelial cell component that appeared to proliferate in a more normal and appropriate fashion. Again the cultures failed to reach plateau phase, indicating further capability for continued growth. A comparison of coculture experiments performed on M3:2/MEM-α and GTSF-2 (Fig. 1C) in RWV demonstrated growth equal to or slightly better than the growth on the primary medium under otherwise identical conditions and at similar intervals in each set of experiments. Maximum cell densities for these cultures were not accomplished; however, due to the slow rate of proliferation, these cultures were terminated at 38–42 days of culture.

Samples from RWV cocultures were harvested at the midpoint and end point of each experiment to test the ability of the cocultured cellular material to proliferate in T-flask cultures as distinct populations while expressing the appropriate phenotypes. Epithelial cells (keratin-positive) and mesenchymal cells (vimentin-positive) were identified and found to proliferate after removal from the RWV and initiation into monolayer culture.

Immunocytochemistry. Immunophenotyping of the cell types used to seed the RWV (Fig. 2) confirmed that the majority of epithelial and mesenchymal cells seeded were keratin- and vimentin-positive, respectively. In addition, the epithelial cells were at various stages of differentiation. Only a small percentage (<2%) of the cells was positive for Factor VIII, an endothelial cell differentiation marker. As described in Materials and Methods and shown in Figure 3A, the cells were seeded onto beads, then grown as a coculture, which led to the formation of large, multilayered, tissue-like masses. Sections of those masses were prepared for immunohistology to verify the cell types being cultured and other attributes of the tissue masses (Figs. 4 and 5; Table IV). Figures 4D and 4E show multicellular aggregates of epithelial cells as outgrowths from the microcarriers, which were seeded with the vimentin-positive fibroblasts (Fig. 4B). Preparation of the specimens sometimes led to the dissociation of mucicarmine-staining epithelial cell aggregates (Fig. 4F) from the tissue masses. It was of interest that an increased percentage ($\geq$10%) of cells present in the masses consisted of endothelial cells that were Factor VIII-positive and growing as patches on the beads (Fig. 4, G and H). Furthermore, a small percentage (1–2%) of cells showed *de novo* expression of the HE3 angioblast antigen (Table IV), which was not present in the seed cultures of mesenchymal cells. This result implied conditions were suitable for some type of mesenchymal stem cell to

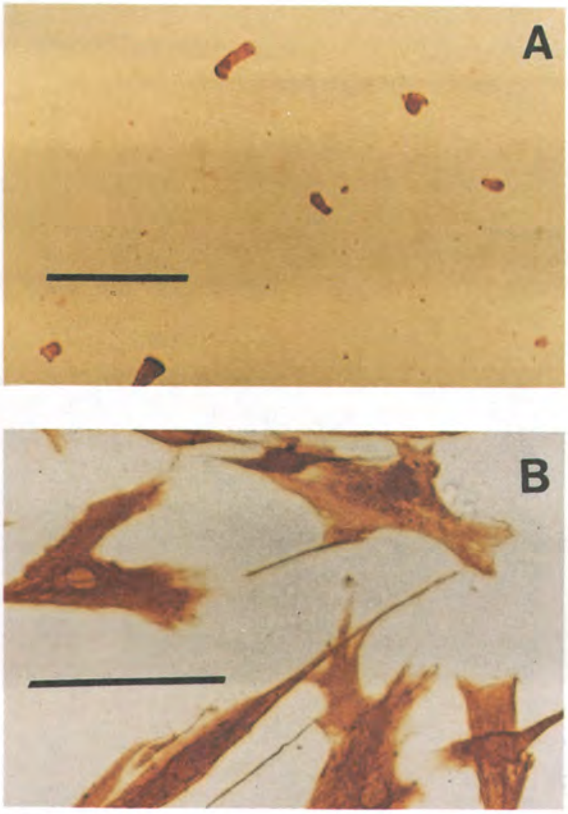

Figure 2. Immunocytochemical characterizations of cultured cells used to seed the RWV. Cells were stained with cell-specific markers. (A) Cytokeratin-positive single, differentiated columnar epithelial cells. (B) Vimentin-stained normal fibroblasts. Bars represent 50 microns.

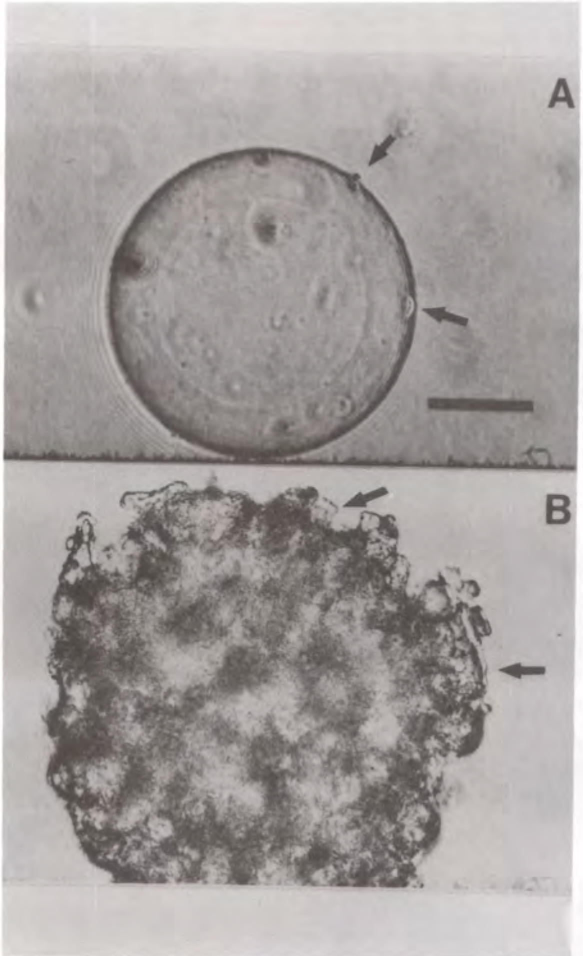

Figure 3. Phase contrast microscopy of cell culture on beads. (A) Initial seeding with cells just starting to attach (arrowheads). (B) After 41 days of growth in the RWV. Note large, multilayered cell mass and appearance of epithelial microvillus-like areas (arrows). Bar represents 50 microns.

differentiate along a lineage of endothelial precursor cells.

Of particular interest were the homogeneous positive staining for villin (Table IV), a cytoskeletal marker found only in epithelial cells of the small intestine and colon, and areas of cells positive for the brush border enzyme, sucrase (Fig. 4I), expressed at the surfaces of some areas of the cell/bead aggregates. As shown in Figure 5, A and B and Table IV, synthesis of basement membrane components was frequently seen at cell-bead-aggregate interfaces.

Fibronectin-positive staining (Fig. 5A, Table IV) was moderate in the early stages of the experiments and became more prominent as the cocultures matured. Laminin-positive responses were seen across time in multiple experiments (Fig. 5B, Table IV), with terminal samples showing the strongest responses. Varying positive responses were recorded for Type IV collagen staining (Table IV) beginning with 13-day samples and continuing through the conclusion of each experiment. Finally, extremely strong positive proteoglycan responses were also obtained from 13-day samples through the termination of the cultures.

Scanning Electron Microscopy. Samples were collected at intervals and prepared for scanning electron microscopy as stated previously. Micrographs taken of 6- to 7-day cocultures showed partial coverage of the microcarriers by normal small intestine mesenchymal cells (Fig. 6, A and B). Additionally, large developing masses of small intestine epithelium were evident, growing on the microcarrier beadpacks. Samples harvested at approximately 12 days of culture contained small microcarrier packs that were totally engulfed in proliferating small intestine epithelium (Fig. 6, C and D). Micrographs of samples at 13 days displayed large tissue-like masses that were entirely covered with small intestine epithelium grown on a base layer of mesenchymal cells (Fig. 6E). In Figure 6F, an area of organized columnar epithelium is prominent. As the cocultures matured into mid- and late-stage cultures, extremely complex tissue-like masses composed of mesenchymal and epithelial cells could be seen in the RWV (Fig. 7, A and B) from approximately 16 days of culture until termination at 41 days. These tissues were assembled from smaller masses now joined by cord-like structures of fibroblasts and covered by epithelial cells several layers deep. In addition, available microcarriers

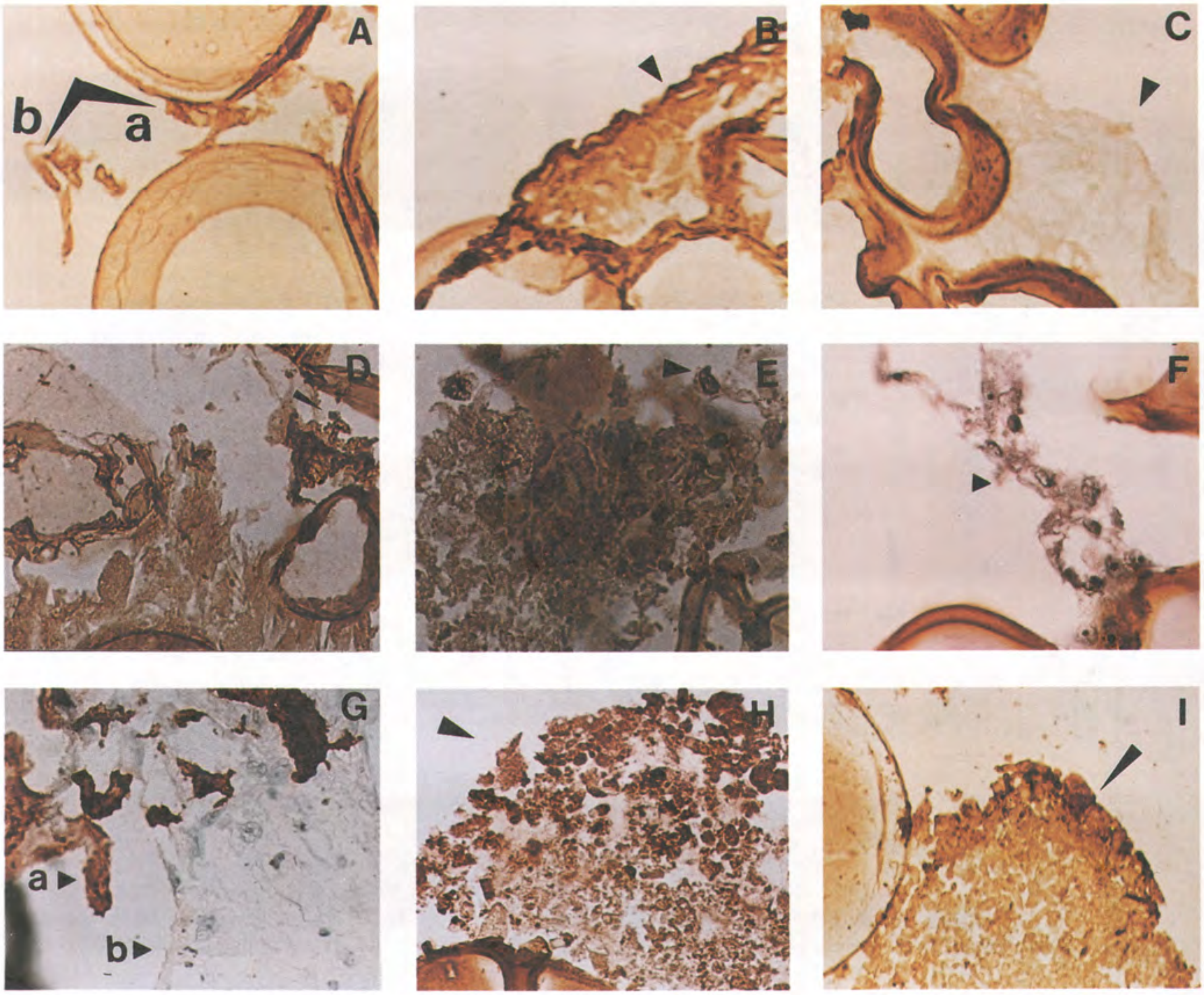

Figure 4. Immunocytochemistry for cell-specific markers. Row 1. Cocultures (A) at 13 days and (B) at 37 days showing vimentin-positive cells free and at the periphery of the cell mass and attached to the bead surface at 37 days. (C) Coculture at 37 days negative control. Primary antibody replaced by normal serum of the same species. Row 2. Cocultures (D) at 13 days and (E) at 37 days showing keratin-staining cell masses attached to the bead surface. Epithelial cells (arrows). (F) Mucicarmine-stained coculture cells showing cell structure at 13 days. Row 3. Cocultures (G) at 13 days and (H) at 37 days stained with Factor VIII for endothelial cells. Note that the majority of cells stain negative at 13 days, whereas the reverse occurs at 37 days. Cocultures (I) at 37 days stained with sucrase-isomaltase (SIYT-1) marker reflecting jejunal villi.

were drawn to the surface of these large masses (Fig. 7, C and D), which were approximately 0.3–0.5 cm in diameter. Finally, columnar epithelium was observed to be growing even in the recessed areas of the microcarrier beadpacks (Fig. 7, E and F).

Discussion

The data presented suggest that a major advance has been made toward constructing a functionally accurate, large-scale, *in vitro*, three-dimensional tissue model of small intestine. The presence of large tissue-like masses that express differentiated epithelial, mesenchymal, and developing endothelial cells affords a multitude of possibilities for cell biological investigations. Functional epithelial cell brush borders with extracellular matrix and basal lamina components represent ordering of tissue and cellular polarity nurtured by the molecular conditions and physical orientations of the culture system. Additionally, this three-dimensional model demonstrates a significantly diminished requirement for complex culture media, which suggests specific cell-cell interactions and the production of paracrine and autocrine factors essential to the growth and development of these fragile tissues.

The growth factor and nutritional differences between GTSF-2 and M3:2 are not insignificant. Although both media are based on Leibovitz-15 (L-15), M3 contains minimal essential medium (MEM Eagle's base), L-Broth (tryptone broth), bovine pituitary extract, hydrocortisone, essential and nonessential amino acids, pentagastrin, epidermal growth factor, and 2% fetal bovine serum. Alternatively, GTSF-2 incorporates

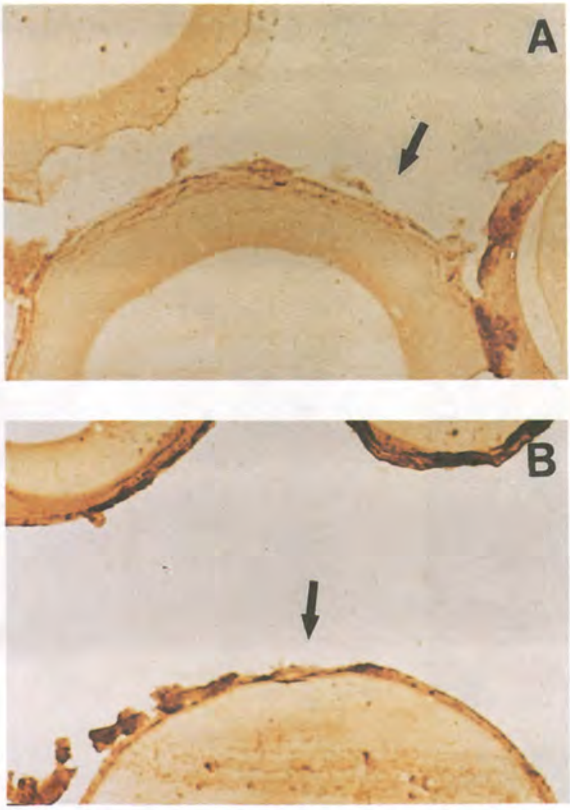

Figure 5. Assays for basement membrane. (A) Fibronectin-stained cells at 25 days and (B) laminin-stained cells at 25 days. Note predominant association with bead surfaces where fibroblasts are found (arrows).

MEM-α, fructose, additional galactose and glucose, vitamins in the form of *i*-inositol, nicotinic acid, and folic acid, bactopeptone broth, and 6% fetal bovine serum. Both media contain equal amounts of transferrin and selenium; however, M3 contains double the amount of insulin. The nature of these factors and cellular interactions provide growth of mesenchymal and epithelial cells alike in the absence of complex media in the RWV culture system. Their roles at the molecular and genetic levels are a subject for further investigations.

The role of basement membranes and extracellular matrix and their relationship to epithelial-mesenchymal development and differentiation are the subjects of considerable research. Current studies indicate that the stromal component exerts a strong and driving influence over developing intestinal mucosa (36–38). Stallmach *et al.* (39) have shown that only organ-specific mesenchyme will produce differentiation in epithelium from a given organ site and that embryonic mesenchyme of the same age but from different organs was ineffective. Studies of rat small intestine development have detailed a shift in the membrane-molecular components of crypt cells as measured by monoclonal antibody binding in fetal versus newborn rat epithelium. Quaroni (40) postulates that the presence of specific markers in the crypts indicates undifferentiated crypt cells, which may be able to perform specific intestinal functions like that of the villus cells. Additional information shows that a single fetal epithelial cell type in the last 2–3 days of gestation may express the function of more than one cell, thereby giving rise to DNA synthesis and proliferation (41).

Cocultures of small intestine produced in the RWV were initiated with adult epithelium composed of dividing and terminally differentiated cells and predominantly adult mesenchymal cells with one exception, a 2-month-old female donor. Levels of epithelial cell (keratin-positive)- and mesenchymal cell-specific (vimentin-positive) immunostaining decreased slightly but consistently over the course of the RWV experiments, while total numbers of cells increased. Levels of endothelial cell (Factor VIII-positive) and epithelial cell (villin-positive) staining remained reasonably constant, while staining for angioblasts (endothelial precursor cells) developed toward the end of each experiment. Functional brush border markers such as sucrase were also present in this coculture system. Other brush-border-specific monoclonal antibodies, kindly supplied

Table IV. Immunoperoxidase-Staining Reactions of Intestinal Cells Grown as Cocultures on Cytodex Beads[a]

Antibodies	Specificity or cell stained	Days grown in RWV (expt. no.)				
		13 (IV)	23 (III)	25 (II)	37 (IV)	41 (V)
Keratin	Epithelial, cytoskeletal	+++	+++	++	++	++
Vimentin	Fibroblasts, some endothelial	+++	+++	++	++	++
Factor VIII	Endothelial	++	+++	++	++	++
Villin	Epithelial, small intestine	+	+	+	+	+
HE3	Angioblasts	—	—	—	++	++
Sucrase	Small intestine, brush border	+++	+	++	++	++
Laminin	Basement membrane	+	+	—	+	++
Fibronectin	Basement membrane	+	+	++	++	—
Proteoglycan	Extracellular matrix	++++	++++	++++	++++	++++
Collagen Type IV	Extracellular matrix	++	+	+	++	ND

[a] Slides were observed and scored on a relative scale as negative (—) to maximum staining; very dark positive for >99% of the cells (++++); + indicates weaker staining for ~25–50% of the cells; ++ indicates moderate staining for greater than 50–75% of the cells.

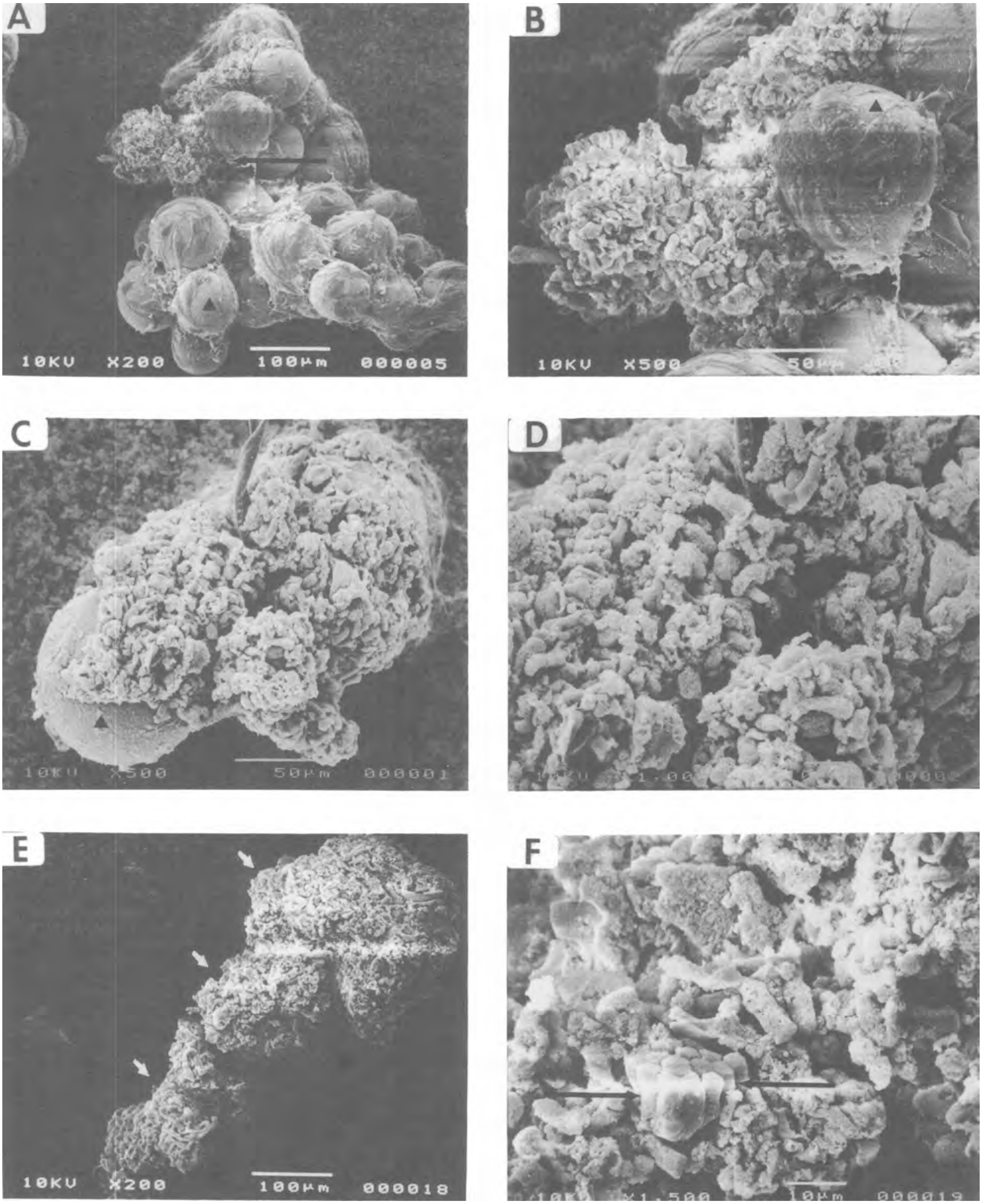

Figure 6. Early-stage scanning electron micrographs of RWV cocultures. (A) Seven-day coculture, ×200. Note the partial covering of mesenchymal cells on the microcarriers at left (triangles) and the mass of growing epithelial cells. (B) Original magnification ×500 micrograph of A. Note the large mass of epithelium. (C) Small beadpack completely covered with epithelium at approximately 12 days, ×500. (D) Original magnification ×1000 micrograph of C. Note the masses of columnar epithelium. (E) A large microcarrier beadpack with a confluent underlayer of mesenchymal cells and total coverage by the epithelium (arrows), ×200. (F) Organized columnar epithelium growing on a confluent beadpack (arrows), ×1500.

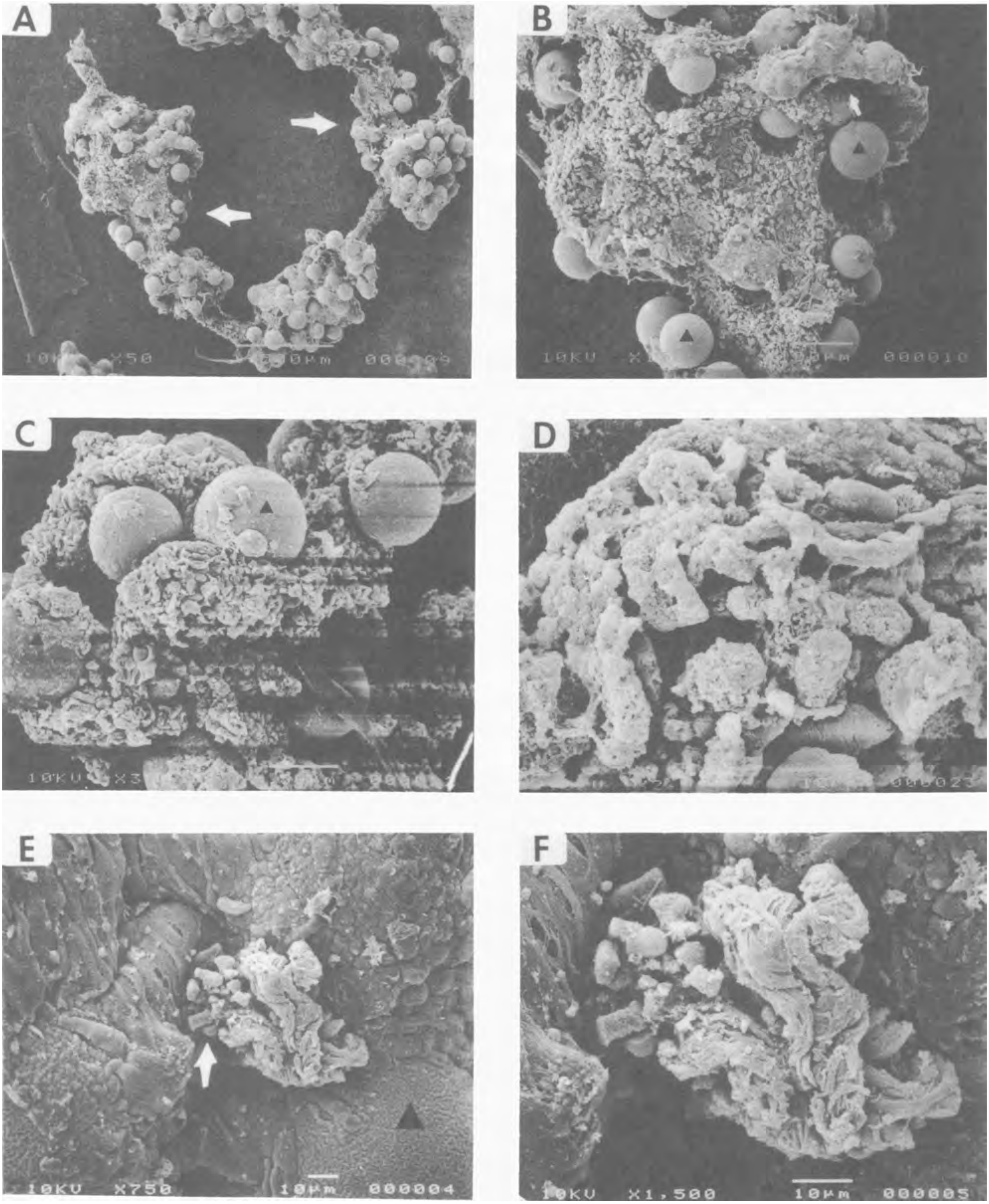

Figure 7. Scanning electron micrographs of mid- and late-stage RWV cocultures. (A) Sixteen-day coculture, ×50. Note the tissue-like masses covered with epithelial cells and joined by cord-like structures of mesenchymal cells. (B) Original magnification ×150 micrograph of A showing a large tissue-like mass. Note the cord-like appearance of the mesenchymal cell covered microcarriers in the upper right (arrow); also free microcarriers drawn to the beadpack (triangles). (C) An epithelial/mesenchymal beadpack at 25 days, ×300, showing dense masses of cells. (D) Original magnification ×200 micrograph of C showing layers of columnar epithelium with thick extracellular secretions. (E) Large beadpack at 37 days, ×750. Note the proliferating columnar epithelium even in small recessed areas between microcarriers (triangle). (F) Original magnification ×1500 micrograph of E. Note the microvillus (furry) appearance of the epithelium.

by Dr. Andrea Quaroni (Cornell University, Ithaca, NY), have similarly proved positive in more recent studies (unpublished results). Collectively, these results indicate that the model, although not histotypically correct, exhibits many facets of functional normal small intestine. Growth conditions, however, may still be skewed slightly in favor of the mesenchymal versus the epithelial cell types. Work will continue on further definition of the correct nutritional and growth-matrix requirements directed toward attaining the best representation possible. Interestingly, all the RWV cocultures exhibited laminin, fibronectin, and Type IV collagen production, as well as large amounts of proteoglycan. Hahn (42) states that only undifferentiated, highly proliferative intestinal epithelial cells (usually fetal) synthesize the above-mentioned proteins. Of these, only laminin appeared to promote differentiation of intestinal epithelial cells. Type IV collagen and fibronectin had no effect.

The literature reviewed and the data obtained here would suggest that this model of human small intestine embodies many aspects of differentiation observed in other *in vitro* and *in vivo* cell and organ models. Primary distinctions would be: (i) the overall scale of the model, (ii) the ability to culture epithelium for long periods (in excess of 40 days) without loss of functional cell markers, and (iii) the ability of the system to respond to extensive analyses and manipulations without the termination of a given experiment. Future experiments will use molecular probes and autoradiographs that will clarify and characterize the potential of this new model system. Of particular interest will be regulation of unique cytoskeletal proteins such as villin, functional markers such as small-intestine-specific enzymes, heat shock proteins, and other markers which may be modulated by simulated microgravity. The fluid dynamic environment of the RWV is extremely quiescent, operating with low turbulence and shear stress, and, therefore, mimics many aspects of microgravity, producing a simulated microgravity effect. This effect can be compared with that of a particle (microcarrier and/or cells) being allowed to fall through a long column of fluid, never reaching the bottom. This mode of operation allows particles of varying sizes (cells and microcarriers) to remain near one another for long periods of time, thus facilitating cell-to-bead bridging. Since the vessel has no agitator or internal mixing device, these bridges are not disturbed, resulting in high-density cell culture that reflects aspects of *in vivo* tissues.

The significance of a complex three-dimensional *in vitro* culture system for the growth of normal small intestine should not be underestimated. The molecular basis and clinical treatment of diseases such as inflammatory bowel disease (Crohn's, ulcerative colitus), malabsorptive syndromes (short-gut syndrome), numerous infectious diseases, and tumors of the small bowel may be investigated with the advent of this new technology. For example, the recent demonstration that the human immunodeficiency virus can replicate in human small intestine and columnar epithelium may be impacted through studies of virus-cell interactions in the RWV culture system (43). This hypothesis is currently being tested. Additionally, general application of this culture model may lead to advances in understanding growth and differentiation in developing organisms and the potential treatment of a myriad of clinical conditions as well as tissue renewal.

This work has been supported by NASA's Microgravity Sciences and Applications Division, Contract No. NAS9-17720. The authors would like to acknowledge the efforts of Luann Kirk for her assistance in the preparation of the document. We would also like to offer special appreciation to Tacey Prewett for her expert technical and administrative assistance. We thank Dr. Alan Elbein for the use of his monoclonal antibody to sucrase. Finally, we thank Dr. Glenn F. Spaulding for his facilitation of this project.

1. Fukamachi H, Mizuno T, and Kim YS. Morphogenesis of human colon cancer cells with fetal rat mesenchymes in organ culture. Experientia **42**:312–315, 1986.
2. Wiens D, Park CS, and Stockdale FE. 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.
3. Sutherland RM. Cell and environment interactions in tumor microregions: The multicell spheroid model. Science **240**:177–184, 1988.
4. Kaye GI, Pascal RR, 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.
5. Buset M, Winawer S, Friedman E. Defining conditions to promote the attachment of adult human colonic epithelial cells. In Vitro **23**:403–412, 1987.
6. Daneker GW Jr, Mercurio AM, Guerra L, Wolf B, Salen RR, Bagli DJ, Steele GD. Laminin expression in colorectal carcinomas varying in degree of differentiation. Arch Surg **122**:1470–1474, 1987.
7. Durban E. Mouse submandibular salivary epithelial cell growth and differentiation in long term culture: Influence of the extracellular matrix. In Vitro **26**:33–43, 1990.
8. Kleinman D, Sharon Y, Sarov I, Inler V. Human endometrium in cell culture: A new method for culturing human endometrium as separate epithelial and stromal components. Arch Gynecol **234**:103–112, 1983.
9. Reid LM, Jefferson DM. Culturing hepatocytes and other differentiated cells. Hepatology **4**:548–559, 1987.
10. Reichman E, Ball R, Gronen B, Friis R. New mammary epithelial and fibroblastic cell clones in coculture form structures competent to differentiate functionally. J Cell Biol **108**:1127–1138, 1989.
11. Haake AR, Lane HT. Retention of differentiated characteristics in human fetal keratinocytes. In Vitro **25**:592–600, 1989.
12. Kabalin J, Poehl D, Stamey TA. Clonal growth of human prostatic epithelial cells is stimulated by fibroblasts. Prostate **14**:251–263, 1989.
13. Chantret I, Barbat A, Dussaulx E, Brattain MG, Zweibaum A. 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.

14. Corps AN, Brown KD. Stimulation of intestinal epithelial cell proliferation in culture by growth factors in human and ruminant mammary secretions. J Endocrinol **113**:285–290, 1987.
15. Pyke KW, Gogerly RL. Murine fetal colon in vitro: Assays for growth factors. Differentiation **29**:56–92, 1985.
16. O'Loughlin EV, Chung M, Hollenberg M, Hayden J, Zahavi I, Gall DG. Effect of epidermal growth factor on ontogeny of the gastrointestinal tract. Am J Physiol **249**:674–678, 1985.
17. Blay J, Brown KD. Functional receptors for epidermal growth factor in an epithelial-cell line derived from the rat small intestine. Biochem J **225**:85–94, 1985.
18. Blay J, Brown KD. Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. J Cell Physiol **124**:107–112, 1985.
19. Blay J, Brown KD. 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.
20. Moyer MP. Mechanisms of tumor initiation and progression. Perspect Gen Surg **1**:71–91, 1990.
21. Moyer MP, Dixon PS, Culpepper AL, Aust JB. The in vitro propagation and characterization of normal, preneoplastic and neoplastic colonic epithelial cells. In: Moyer MP, Poste GH, Eds. Colon Cancer Cells. San Diego: Academic Press, pp85–136, 1990.
22. Moyer MP. Culture of human gastrointestinal epithelial cells. Proc Soc Exp Biol Med **174**:12–15, 1983.
23. Shamsuddin A. Colon organ culture as a model for carcinogenesis. In: Moyer MP, Poste GH, Eds. Colon Cancer Cells. San Diego: Academic Press, pp137–153, 1990.
24. Goodwin TJ, Jessup JM, Sams CF, Wolf DA. In Vitro Three-Dimensional Modeling. Annual report of Johnson Space Center Research and Technology, pp156–157, 1988.
25. Goodwin TJ, Jessup JM, Wolf DA. Morphologic differentiation of colon carcinoma cell lines HT-29 and HT-29KM in rotating-wall vessels. In Vitro **28A**:47–60, 1992.
26. Schwarz RP, Wolf DA, Trinh T. Rotating cell culture vessel. U.S. Patent No. 5,026,650.
27. Schwarz RP, Goodwin TJ, Wolf DA. Cell culture for three-dimensional modeling in rotating-wall vessels: An application of simulated microgravity. J Tiss Cult Meth **14**:51–58, 1992.
28. Tsao YD, Goodwin TJ, Wolf DA, Spaulding GF. Responses of gravity level variations on the NASA/JSC bioreactor system. The Physiologist **35**:S49–S50, 1992.
29. Moyer MP. Methods for propagation and characterization of human GI and other cells for study of HIV. J Tiss Cult Meth **13**:107–116, 1991.
30. van Wezel AL. Microcarrier culture of animal cells. In: Kruse PF, Paterson MK, Eds. Tissue Culture: Methods and Applications. New York: Academic Press, pp372–377, 1973.
31. Woodcock-Mitchell J, Eichman R, Nelson WG, Sun T. Immunolocalization of keratin polypeptides in human epidermis using monoclonal antibodies. J Cell Biol **95**:580–588, 1982.
32. Vogel AM, Gown AM. Monoclonal antibodies to intermediate filament proteins. In: Shay JW, Ed. Cell and Muscle Motility. New York: Plenum Publishing, Vol **5**: pp379–402, 1984.
33. Shima M, Yoshioka A, Nakai H, Tanaka I, Fujiwara T, Terada S, Imai S, Fukui H. Factor VIII polypeptide specificity of monoclonal anti-factor VIII antibodies. Br J Haematol **70**:63–69, 1988.
34. Zweibaum A, Hauri H, Sterchi E, Tret IC, Haffen K, Bamat J, Sordat B. Immunohistological evidence, obtained with monoclonal antibodies of small intestinal brush border hydrolases in human colon cancers and foetal colons. Int J Cancer **34**:591–598, 1984.
35. Luna LG (Ed.). Histologic staining methods. In: American Registry of Pathology, 3rd ed. New York: Armed Forces Institute of Pathology, 1968.
36. Haffen K, Kedinger M, Simon-Assmann P. Mesenchyme-dependant differentiation of epithelial progenitor cells in the gut. J Pediatr Gastroenterol Nutr **6**:14–23, 1987.
37. Kedinger M, Simon-Assmann PM, Lacrois B, Marxer A, Hauri HP, Haffen K. Fetal gut mesenchyme induces differentiation of cultures intestinal endodermal and crypt cells. Dev Biol **113**:474–483, 1986.
38. Kedinger M, Haffen K, Simon-Assmann P. Intestinal tissue and cell cultures. Differentiation **36**:71–85, 1987.
39. Stallmach A, Hahn U, Merker HJ, Hahn EG, Rieken EO. Differentiation of rat intestinal epithelial cells is induced by organotypic mesenchymal cells in vitro. Gut **30**:959–970, 1989.
40. Quaroni A. Crypt cell development in newborn rat small intestine. J Cell Biol **100**:1601–1610, 1985.
41. Quaroni A. Development of fetal rat intestine in organ and monolayer culture. J Cell Biol **100**:1611–1622, 1985.
42. Hahn U. Extracellular matrix proteins in small intestinal cell cultures. Scand J Gastroenterol **23**(suppl 151):70–78, 1988.
43. Moyer MP, Hout RI, Ramirez A Jr, Joe S, Meltzer MS, Gendelman HE. Infection of human gastrointestinal cells by HIV-1. AIDS Res Hum Retroviruses **6**:1409–1415, 1990.