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DEFINING CONDITIONS TO PROMOTE THE ATTACHMENT OF ADULT HUMAN COLONIC EPITHELIAL CELLS

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SUMMARY

An improved method for the attachment and growth of normal human colonic epithelial cells from minute 1 to 3-mm³ biopsies has been developed. This yields four times as many cultured cells per biopsy than older methods, with a success rate of 97% in a series of 29 biopsies. Fetal bovine serum was eliminated from the medium, the medium pH was decreased to 6.7, the oxygen tension in the incubator was decreased to 3%, and the NCTC 168 medium was supplemented with ethanolamine, phosphoethanolamine, hydrocortisone, ascorbic acid, transferrin, glutamine, insulin, epidermal growth factor, pentagastrin, and deoxycholic acid. The best substrate for cell attachment was a mixture of ungelled collagen I and bovine serum albumin. This substrate was better than the identical mixture with fibronectin added, fibronectin alone, a thin gelatin film, collagen IV with or without fibronectin, and basement membrane preparations from four different cell lines.

Key words: normal colonic epithelial cell culture.

INTRODUCTION

The extended in vitro monolayer culture of human colonic cells from adults is a goal of this laboratory. We first developed a simple method to place normal human colonic epithelial cells from minute (1 to 3 mm³) colonoscopic biopsies into monolayer culture (7) using a highly enriched medium containing 15% fetal bovine serum, and plates coated with gelatin for efficient cell attachment. The cultures were used to identify patients at high risk for colon cancer. These patients had colon cells that exhibited abnormally high proliferation rates (7). However, the culture procedures yielded few cultured cells. Each biopsy, from a series of 34 patients, yielded an average of 16 colonies with a mean colony size of 331 cells, about 5300 cells total per patient. An improved yield of viable cells was necessary before more extensive experiments could be undertaken. In this study the parameters varied to improve cell attachment were: digestion conditions, oxygen tension, medium pH and constituents, and the culture substrate.

MATERIALS AND METHODS

Culture of Normal Colonic Epithelial Cells

Protocol I [original procedure (7)]. Colonic biopsies were taken either from patients genetically at high risk for the development of colon cancer because of a family history of colon cancer or from patients symptomatic because of the development of adenomas. Biopsies were received from endoscopy within 30 min of removal from the patient. Biopsies of about 1 to 3 mm³ were taken with a biopsy forceps of only the mucosal layer which consists of approximately 50% epithelial cells: 50% lymphocytes. Very few fibroblasts are present lining the epithelial layer of the colonic crypts. The biopsies do not reach the muscle layer. The biopsy is washed three times with a wash medium (8) of Dulbecco's modified Eagle's medium supplemented with 25 m*M* HEPES buffer, pH 7.3, with NaCl level decreased to allow addition of HEPES. The wash medium contained 1.4 mg/ml bovine serum albumin (BSA) (lypholized but containing globulins, Sigma, St. Louis, MO), 250 U/ml penicillin, 250 μg/ml streptomycin, 5 μg/ml Fungizone, 10 μg/ml tetracycline, 100 μg/ml amakacin, 150 μg/ml chloramphenicol, and 100 μg/ml Gentamycin. The biopsy was minced finely with two scalpels, then placed in 3 ml of the antibiotic-containing wash medium and 1 ml each of hyaluronidase (type V, Sigma, 1000 U/ml), collagenase (type IV, Worthington, Freehold, NJ, 4 mg/ml), and neuraminidase (type V, Sigma, 4 U/ml). The minced biopsy was digested for 10 min at 37° C and 1 h in a 15-ml conical centrifuge tube on a blood rotator at room temperature. The digest was then washed once by centrifugation at 100 ×*g* for 5 min at room temperature, resuspended in 10 ml of growth medium (*see* below), repelleted, then resuspended in 1.2 ml of growth medium, and 0.2 ml of the mixed, resuspended digest

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was plated on each of six 35-mm petri dishes coated just before use with 0.1% gelatin in water. One milliliter of gelatin was added per dish, then aspirated so that a very thin, unscratched film was present per dish. The plated partial digest was allowed to adhere undisturbed in a 5% CO_2 environment in the incubator for 45 min. Then 1 ml of growth medium was added to the side of each dish, being careful not to disturb the plated cells. Growth medium consisted of NCTC 168 (K. C. Biologicals, Lenexa, KS), pH 7.2, supplemented with 15% fetal bovine serum (heat inactivated by 56° C for 30 min with shaking), 1 μg/ml hydrocortisone, 38 μg/ml ascorbic acid, 1.7 μg/ml transferrin, 4 m*M* glutamine, 0.1 U/ml insulin (regular, Lily Research Labs, Indianapolis, IN), 60 μg/ml Gentamycin, 10^{-8} *M* selenous acid, 30 ng/ml epidermal growth factor (Collaborative Research, Waltham, MA), 5 μg/ml pentagastrin, and 10^{-8} *M* deoxycholic acid. The formulation of the medium contained 2.2 m*M* $CaCl_2$. All ingredients with source not designated were purchased from Sigma Chemical Co.

Protocol 2. Our original procedure (protocol 1) was modified in the following ways. The room temperature digestion time was lengthened to 3 to 5 h, and 0.1 ml of a 20% solution of the mucolytic agent Mucomyst (acetylcyteine, Mead-Johnson, Evansville, IN) was added to the 6 ml digest. The digestion was carefully monitored and the digestion ended when cells were observed to clump, probably due to release of DNA from lysed cells. The partial digests were plated on a prepared substrate: 30 μg/ml collagen I (Vitrogel 100, Collagen Corp., Palo Alto, CA), 10 μg/ml of BSA (crystallized and lypholyzed globulin-free), and 10 μg/ml of fibronectin (FN) (Bethesda Research Laboratories, Gaithersburg, MD) was prepared in serum-free, calcium-free NCTC 168 medium, and 1 ml was added to each 35-mm petri dish and placed in a CO_2 incubator for 2 to 24 h. Immediately before plating the digest (a mixture of partially digested colonic crypts and clumps of epithelial cells) the substrate mixture was carefully aspirated off, leaving a thin film. The pelleted colonic epithelial digest was washed once as in protocol 1, then resuspended in serum-free growth medium. The volume of the resuspension medium was altered so that 0.1 ml was added for each dish to be plated, typically 8 to 12 plates. The cells were allowed to attach for 1 h. Then 1 ml of

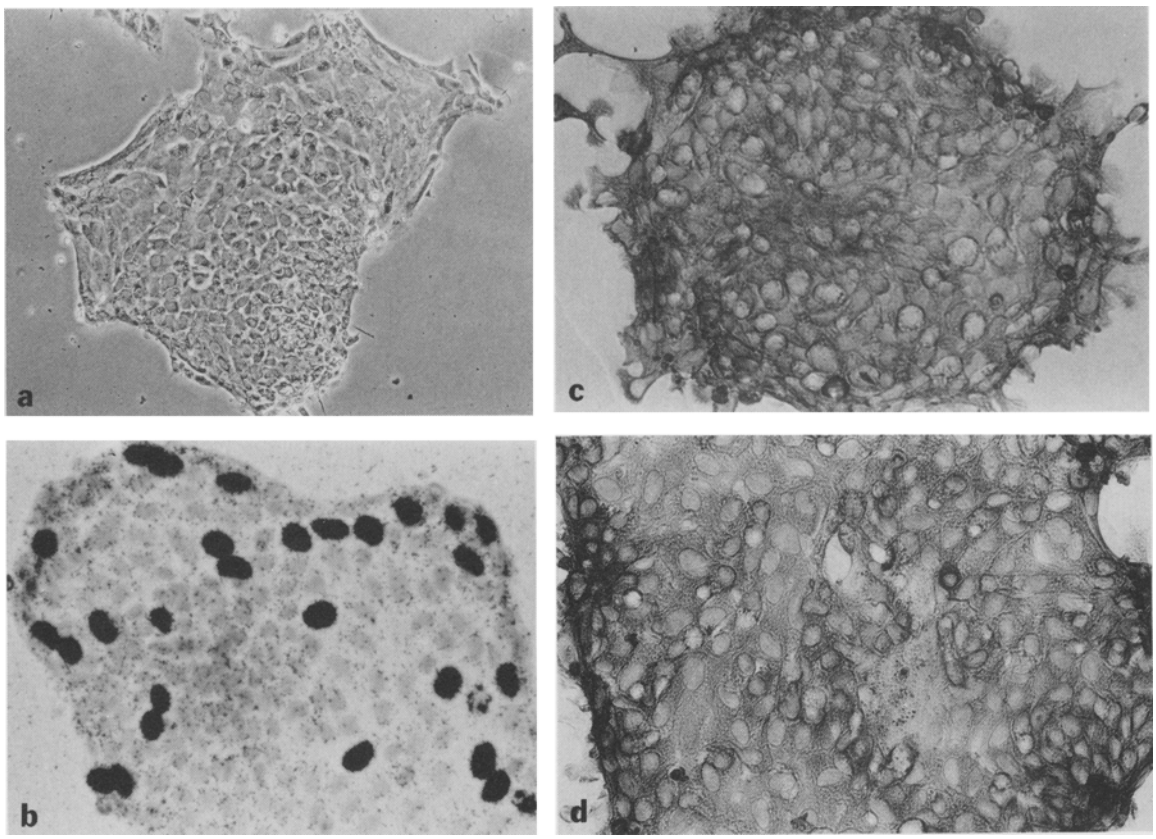

FIG. 1. *a*, Live human colonic epithelial cell colony prepared by protocol 2, 1 d after plating in serum-free medium on a collagen I-BSA substrate. Phase microscopy. Final ×107. *b*, Autoradiograph of colonic epithelial cell colony prepared by protocol 2 showing [^{3}H]TdR-labeled nuclei. Unlabeled nuclei are visible due to hematoxylin stain. Final ×213. *c*, Colonic epithelial colony prepared by protocol 2, after methanol fixation and indirect immunoperoxidase staining with anticytokeratin monoclonal antibody cam-5 (Methods). The cells were not counterstained so all of the staining is due to the intensity of the immunoperoxidase reaction. Final ×200. *d*, Colonic epithelial colony from the same digest as the colony in *c*, stained for the presence of cytokeratin filaments by anticytokeratin monoclonal antibody 4.62 (Methods). The cells are not counterstained so all of the staining is due to the intensity of the immunoperoxidase reaction. Final ×260.

growth medium was added, and the plates placed in an incubator at 3% O_2:5% CO_2 for 24 h to allow migation onto the plate of the epithelial monolayer from the attached groups of epithelial cells (Fig. 1). The O_2 level was controlled by the O_2 electrode, supplied by the manufacturer, in a Hereaus incubator.

The growth medium used in protocol 2 was NCTC 168 modified as in protocol 1 with the following changes: the calcium level was reduced to 0.1 m*M*, and 0.1 m*M* of both ethanolamine and phosphoethanolamine were added. No serum was added.

Autoradiography. One milliliter of growth medium containing 5 μCi of [^{3}H]thymidine (TdR) (20 Ci/mmol) was added after the plated digest had been allowed to attach for 1 h. The dishes were incubated for 24 h, then the medium was carefully aspirated off, and the cells fixed twice, for 10 min each time, at 25° C in methanol. The dried dishes were coated with undiluted NTB2 emulsion (Kodak), exposed for an average of 10 d, then developed, counterstained with hematoxylin, and the fraction of labeled epithelial cells counted per colony per dish. All cells were read per plate using an inverted microscope at ×100. One scorer read an entire experiment to eliminate any possible interindividual scoring. A reticle within one of the eyepieces contained a micrometer which was used to demarcate the section of the colony being read.

Basement membrane preparations. The rat liver epithelial line ARL-18 and the rat liver endothelial cell line RLC-4A were the gifts of Dr. Paul Higgins, Albany Medical School. The capillary endothelial cell strain (BACE) was the gift of Dr. Julio Alessandri, National Institutes of Health, Bethesda, MD, and the bovine pulmonary artery endothelial cell line CPA was purchased from the American Tissue Type Collection, Rockville, MD, and cultured in conditioned medium as described in the information supplied with the line. The ARL-18, RLC-4A, and BACE cells were grown in Dulbecco's modified Eagle's medium buffered with 25 m*M* HEPES, pH 7.3 (NaCl level adjusted), and supplemented with 15% newborn bovine serum. To prepare basement membranes, cells were seeded at 0.5× saturation density, and 50 μg/ml ascorbic acid was added fresh daily. The cellular layer was washed three times with phosphate buffered saline (PBS), then removed with 0.025 *M* NH_4OH and washed with PBS. The basement membrane remaining attached to the dish was allowed to air dry under sterile conditions in a laminar flow hood with the covers slightly ajar. The dishes were wetted with 1 ml growth medium, which was carefully pipetted off just before use.

Other Reagents

Fibronectin was first purchased from Bethesda Research Laboratories and finally from Sigma. When FN was tested without BSA and collagen I, 1 mg was dissolved at room temperature in 1 ml growth medium for 30 min, then aliquoted into 35-mm dishes containing 1 ml growth medium, and allowed to evaporate to dryness. Collagen IV and laminin were also purchased from BRL. One milligram of collagen IV was dissolved in 0.1 *M* acetic acid, and appropriate dilutions were added to 1 ml of sterile water in 35-mm dishes, and allowed to evaporate to dryness. Each dish coated with either fibronectin or collagen IV was wetted with 1 ml growth medium, which was then aspirated off just before use.

Cytokeratin Staining

Epithelial cultures were fixed in methanol, as for autoradiography above, then indirect immunoperoxidase assay was performed exactly as described (9) using as a primary antibody the following monoclonals against cytokeratins: anticytokeratins AE1 and AE3 (18) (gift of Dr. T.-T. Sun, New York University Medical School), human anticytokeratin cam-5 for molecular weight 39K, 43K, and 50K (Becton-Dickinson, Mountain View, CA), and anticytokeratin 4.62 (ICN ImmunoBiologicals, Lisle, IL). Each monoclonal was used at a 1:100 dilution in PBS containing 0.1% BSA. The negative control was IgG1, MOPC-21 (9).

RESULTS

Better Growth Under Lower O_2 Tension

As a first step, the effect of growth under lowered oxygen tension was assayed. Equal aliquots of a biopsy were digested and plated using the original protocol 1 (Methods) in six dishes. Each dish was labeled with 5 μCi/ml [^{3}H]TdR. Three dishes were arbitrarily chosen and placed in an incubator at 3% O_2:5% CO_2, while the

TABLE 1

IMPROVED PLATING OF COLONIC EPITHELIAL CELLS[a]

A. at Low O_2 Tension

O_2 Concentration	[^{3}H]TdR-Labeled Cells, mean ± SE	Total Cells
21% (in air)	24.2 ± 3.2%	1802
	$P<0.05$	
3% (reduced tension)	32.0 ± 1.4%	4848

B. at pH 6.7

pH of Medium	[^{3}H]TdR Labeled Cells, Mean ± SE	Cells per Colony, Mean ± SE	Number of Colonies
6.2	4.3 ± 1.1%	109 ± 22	9
	$P<0.05$		
6.7	6.9 ± 1.0%	248 ± 27	11
		$P<0.0005$	
7.6	6.9 ± 1.3%	112 ± 17	15

[a] For the experiments in *A*, the biopsy was digested and plated on gelatin films as in protocol 1 (Methods). The digest was plated onto six dishes, and three arbitrarily chosen and placed at 3% O_2:5% CO_2, while the others were placed in a standard 5% CO_2 incubator in air. For *B*, the digestion and plating were performed by protocol 2 (Methods), and a portion of a digest was plated in six dishes at the three different pH values. After attachment, each culture was [^{3}H]TdR-labeled for 24 h, processed for autoradiography, and counted (Methods).

other three were placed in a standard 5% CO_2 incubator in air (21% O_2). After 24 h of labeling, all cultures were fixed and processed for autoradiography. Under the lower oxygen tension (Table 1 *A*) over twice as many cells plated, and of these a statistically significant higher fraction ($P < 0.05$) underwent DNA synthesis. No other oxygen tensions were tested.

Development of Protocol 2

The original biopsy was minced even finer than the usual 0.5 to 1 mm^3, the length of digestion increased to 3 to 5 h from 1 h, and 0.1 ml of a 20% solution of the mucolytic agent mucomyst (acetylcysteine) was added to the 6-ml digest (Methods). The growth medium was made serum-free, 0.1 m*M* phosphoethanolamine and 0.1 m*M* ethanolamine added, and the $CaCl_2$ concentration decreased to 0.1 m*M*. Colonic epithelial cells plated very poorly in this new medium on the gelatin films used in protocol 1, so a new substrate of FN-collagen I-BSA was chosen arbitrarily. This substrate had been effective for epithelial cell plating under serum-free conditions (13,19). Seven biopsies were processed by protocol 2 and plated on either gelatin films or the FN-collagen I-BSA substrate. To allow comparisons between biopsies, the number of colonies from each biopsy plated on FN-collagen I-BSA was set at 100%, and the number of colonies on gelatin then calculated as a percentage of the number on FN-collagen I-BSA. A mean value of 1 ± 1% (SE) plating occurred on gelatin on serum-free conditions while 60 ± 20% plating on gelatin was observed when 15% fetal bovine serum was added. Therefore, under serum-free conditions the FN-collagen I-BSA substrate was 100 times better than the gelatin substrate.

Effect of Lowered pH

The pH of the colonic lumen has been reported to vary between 6 and 8. Therefore media at various pHs was tried, first using human colon carcinoma cells from a surgical resection. The carcinoma cells were digested and plated using protocol 2, except that the medium pH was adjusted with either HCl or NaOH to pH 6.1, 6.7, 7.3, or 8.2. Then the cells were labeled for 24 h with [^{3}H]TdR, and autoradiographed. The [^{3}H]TdR-labeling indexes at the different pHs were 32.6 ± 1.2% at pH 6.1, 49.3 ± 7.2% at pH 6.7, 32.2 ± 6.5% at pH 7.3, and 22.5 ± 0.3% at pH 8.2, showing an optimum at pH 6.7.

This experiment was repeated using a narrower pH range for normal cells. A biopsy was digested and plated by protocol 2 using media prepared at pH 6.2, 6.7, and 7.6. A statistically greater number of cells plated at pH 6.7 than at either pH 6.2 or 7.6 and cells at pH 6.7 displayed greater replication than at pH 6.2 (Table 1 *B*). All subsequent assays were performed at pH 6.7.

Assay of Reproducibility of Plating and Yield of Cultured Epithelial Cells Under Serum-Free Conditions Following Protocol 2

Three biopsies were plated in eight petri dishes each using protocol 2. The data are shown as the mean number of cells per colony per plate (mean ± SD), the total number of cells per plate, and the total number of colonies per plate (Table 2). The mean number of cells per colony was averaged per petri dish plated and yielded similar values within the SD for each biopsy: 144 ± 22 cells for patient 111, 153 ± 24 cells for patient 113, and 194 ± 26 for patient 104. The average of these values was 164 ± 24 cells/colony, about one-half the mean size of the colonies obtained with protocol 1 (331 ± 67 cells), which was not surprising with the longer digestion time and more

TABLE 2

REPRODUCIBILITY OF COLONIC EPITHELIAL CELL PLATING UNDER SERUM-FREE CONDITIONS

Plate No.	Cells per Colony	Cells per Plate	Colonies per Plate
Patient 111			
1	130 ± 21	1560	12
2	147 ± 20	1911	14
3	107 ± 16	1611	15
4	171 ± 31	2569[a]	15
5	136 ± 16	1909	14
6	149 ± 25	2378	16
7	169 ± 26	2540[a]	15
8	140 ± 24	1399	10
Totals	144 ± 22 mean values	15 877 cells 1985 ± 459 mean ± SD SD = 18% of mean	111 colonies 14 ± 2 mean ± SD
Patient 113			
1	139 ± 21	1526	11
2	165 ± 17	1654	10
3	135 ± 16	2164	16
4	144 ± 33	2456[a]	17
5	136 ± 30	1626	12
6	150 ± 21	2101	14
7	190 ± 26	1713	9
8	162 ± 28	1623	11
Totals	153 ± 24 mean values	14 863 cells 1858 ± 336 mean ± SD SD = 23% of mean	100 colonies 13 ± 3 mean ± SD
Patient 104			
1	127 ± 8[a]	1777[a]	14
2	199 ± 25	4587	23
3	219 ± 33	3730	17
4	208 ± 24	4988[a]	26
5	167 ± 23	3177	19
6	185 ± 25	3639	20
7	282 ± 49	4225	15
8	165 ± 21[a]	3950	24
Totals	194 ± 26 mean values	30 073 3759 ± 981 mean ± SD SD = 26% of mean	158 20 ± 4 mean ± SD

[a] Indicate values that differ from the mean values more than the SD. The majority of values do fall within the SD. Each biopsy was digested and plated by protocol 2 (Methods), labeled with [^{3}H]TdR for 24 h, autoradiographed in situ, and counted.

extensive mincing employed in protocol 2. The values on 22 of 24 plates were identical to the mean value within the limits of the averaged SD for each digest, indicating the reproducibility of plating.

To assess the reproducibility of the number of colonic epithelial cells cultured per plate, the mean (± SD) of the total cells in monolayer culture per plate was calculated for each biopsy. The number of cells per plate varied no more than the SD in 19 of the 24 plates. The SD was calculated as a percent of the mean value for each biopsy, giving values respectively of 18, 23, and 26% for biopsies 111, 113, and 103. The SD was not large, being 22% of the mean cell number cultured for these three biopsies, again indicating reproducibility of plating.

From these biopsies an average of 123 ± 18 colonies were cultured per biopsy, approximately eight fold more colonies than obtained with the high-serum plating method on gelatin (15.6 ± 1.4 colonies). Thus protocol 2 gave a mean of 20,271 epithelial cells cultured per biopsy whereas the original protocol 1, which used a high-serum, high-calcium medium with plating on a gelatin substrate, yielded about one-fourth as many cultured cells, a mean of 5164 cells per biopsy.

Identification of the Cultured Cells as Epithelial

The colonic biopsies are relatively shallow, taking only the mucosal layer of the gut, which is composed of a monolayer of epithelial cells indented into test tubelike crypts. The epithelial cells comprise about 50% of the total cells of the mucosa. The majority of the remaining cells are the lymphocytes of the lamina propria, which are found between the crypts. A few pericryptal fibroblasts are found which are a small minority of the total cells. The biopsies do not reach into the muscle layer. Partial digestion of the biopsy with rotation of the test tube releases the lymphocytes from the intercryptal spaces, and the low-speed centrifugation removes most of the single cells, including the lymphocytes. Only the epithelial cells from the digest adhere as clusters of cells, and then form flat epithelial patch colonies (Fig. 1 *a*). Autoradiography in situ of these cells is readily performed and scored as [^{3}H]TdR-labeled and unlabeled nuclei are easily distinguished (Fig. 1 *b*). In our previous study (7) we performed electronmicroscopy of the epithelial colonies. The cultured cells exhibited characteristic epithelial substructures, such as brush borders and junctional complexes between cells; goblet cells were also seen.

In this study, we verified the epithelial nature of the cultured cells by assaying the binding of anticytokeratin monoclonal antibodies by indirect immunoperoxidase reaction (Methods). Four anticytokeratin monoclonals were used: AE1, AE3 (18), cam-5 (Fig. 1 *c*) and 4.62 (Fig. 1 *d*), and a IgG1 isotype negative control, MOPC-21 (9). The first three monoclonals bind to several keratin polypeptides, whereas the 4.62 monoclonal is reported by the supplier to bind only to cytokeratin 19. The epithelial colonies shown were not counterstained, so all of the darkness in the black and white photos is due to the intensity of the golden brown immunoperoxidase staining. The negative control was unstained. Some of the epithelial cells exhibited much greater intensity of cytokeratin staining than others, in particular the cells stained with monoclonal 4.62 at the middle of Fig. 1 *d*. The elongated cells at the edges of the colonies also contained cytokeratin filaments (Fig. 1 *c*).

Determination of Optimal Concentrations of Collagen I, Fibronectin, and Collagen IV for Colonic Cell Plating

The FN-collagen I-BSA substrate was chosen arbitrarily because it had been used successfully by other investigators for epithelial cells (13,19). We decided to optimize the concentrations of the three components for colon cell attachment and to test whether the addition of collagen IV would improve plating.

In experiments on two biopsies shown in Fig. 2, an optimum collagen I concentration of 15 to 30 μg/dish was found ($P < 0.05$ compared with mean, one-tailed t test). The highest concentration of collagen I tested, 300 μg/dish, inhibited attachment in both experiments ($P < 0.05$). The [^{3}H]TdR-labeling index of the plated cells was identical within statistical variation at each concentration of collagen I tested, from 3 to 300 μg collagen I/35-mm dish (Fig. 2). Therefore, the higher concentrations of collagen I inhibited only attachment of the plated cells, but not their subsequent growth.

Six different ways to prepare collagen I-coated dishes were then tested (Table 3). Thirty microgram of collagen I was added directly to medium with and without BSA or FN, or directly to H_2O. The largest number of cells were cultured on collagen I mixed with BSA, whereas about

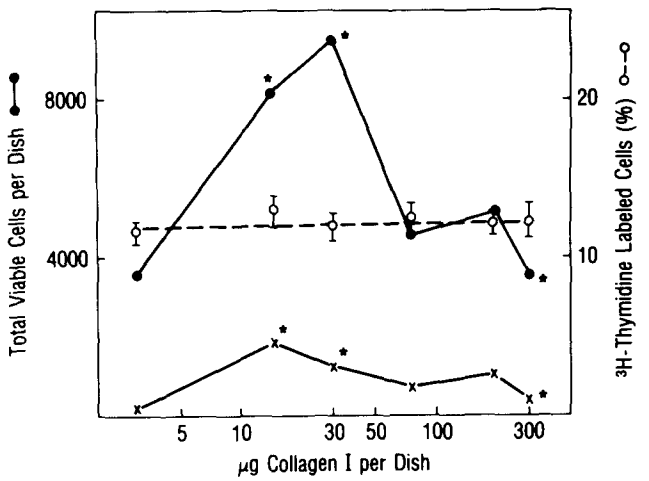

FIG. 2. Optimal collagen I concentration for attachment. Soluble collagen I (Methods) was diluted either into additive-free growth medium (*upper curve*, o—o) or sterile water (*lower curve*, x—x) and placed for 2 h in a CO_2 incubator. The liquid was removed, leaving only a thin, unscratched film, and equal aliquots of a colonic biopsy digest added to each set. The cells were labeled with [^{3}H]TdR for 24 h, autoradiographed, and each colony counted. Only the [^{3}H]TdR data (mean ± SE) is shown for the collagen I in medium experiment (o—o). A similar lack of modulation of [^{3}H]TdR was obtained with the other collagen preparation (not shown). The values for numbers of attached cells significantly ($P < 0.05$) different from the mean are indicated by *asterisks*.

half as many cells plated on collagen I alone diluted into medium or H_2O. Surprisingly, four times as many cells plated on the mixture of collagen I and BSA alone than when FN was added. None of these collagen preparations was gelled by addition of NaOH before dilution. However, the medium is phosphate and bicarbonate buffered, so the collagen was probably neutralized during incubation but was at too low a concentration to gel. No gel was observed. Gelation of the collagen I according to the supplier's instructions (21) yielded a very poor substrate to which no colonic cells attached (Table 3). Lack of attachment had been observed in previous studies using gelled substrates with the high serum medium (data not shown). Two explanations for the lack of attachment were possible—the physical state of gelated collagen or the high concentration of collagen I. Therefore, an identical concentration of collagen I to that gelled (2.4 mg) was not neutralized, but allowed to dry to a film on the dish before use. Some colonic epithelial cells attached to this dried substrate but none attached to the gelled collagen I, showing that a gelled substrate inhibited attachment. About three times as many cells attached to the 30 μg of dried collagen I compared to the 2.4 mg of dried collagen I. This inhibition by 2.4 mg of collagen I is not surprising as the dose-response curve in Fig. 2 showed that 300 μg of collagen I/dish was inhibitory to plating compared to 15 to 30 μg.

The observation that the addition of FN to collagen I and BSA made the substrate poorer was unexpected. Possibly the FN concentration of 10 μg/plate was not optimal for attachment. Cells were plated on FN at concentrations of 1 to 100 μg/plate to determine the optimum for plating. A broad curve was observed with similar levels of binding between 10 and 100 μg/35-mm dish (Fig. 3). Similarly, the [^{3}H]TdR-labeling indexes for cultures at 1 to 100 μg FN averaged to 16.1 ± 1.3%, almost identical values (not shown). The 10 μg of FN/plate used in the mixed substrate was within the broad optimal range.

TABLE 3

COMPARISON OF METHODS OF PREPARATION OF COLLAGEN I-COATED PETRI DISHES[a]

Collagen I Preparation	Cells Cultured per Plate	Colonies per Plate	[^{3}H]TdR Labeling Indexes mean ± SE
30 μg into 1 ml medium	4887	29	29.1 ± 1.3
30 μg + 10 μg BSA/1 ml medium	10 584	63	31.3 ± 1.4
30 μg, 10 μg BSA, 10 μg FN	2398	22	27.8 ± 1.8
2.4 mg gelled by neutralization	0	-	-
2.4 mg dried	950	12	33.0 ± 2.5
30 μg into 1 ml H_2O, dried	3373	26	33.7 ± 1.4

[a]The first three preparations were made up in NCTC 168 without any additives (no serum, no calcium, no growth supplements). One milliliter of each mixture was added per dish and placed in the incubator for 2 to 6 h; then the medium was carefully aspirated and aliquots of the digest applied. The collagen I gel was neutralized by NaOH and PBS was present at 1X final concentration. The collagen I solution was allowed to dry without neutralization for the fifth preparation. In the last preparation, 30 μg of collagen I was diluted into H_2O and allowed to dry without neutralization.

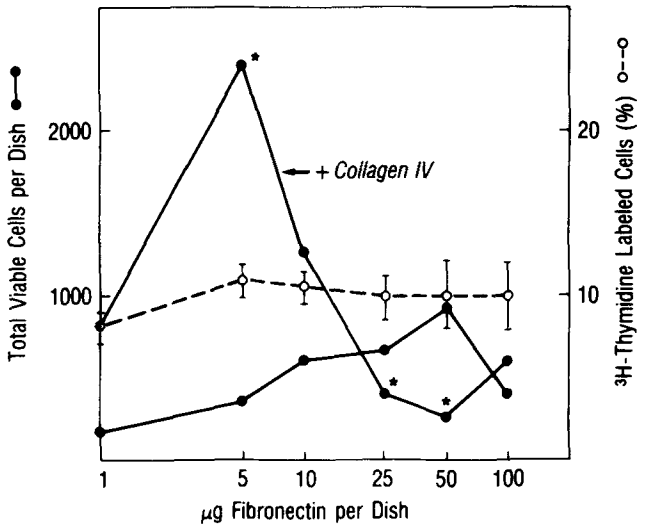

FIG. 3. Effects on colonic cell attachment and replication of varying fibronectin concentration alone or added to collagen IV. One milligram of fibronectin was diluted into 1 ml of growth medium and dilutions from this stock made into 35-mm dishes containing 1 ml of growth medium. In the *upper curve* labeled "+ collagen IV" the fibronectin dilutions were made into 35-mm dishes containing 10 μg of collagen IV evaporated to dryness. Equal aliquots of a colonic biopsy were added to the dishes. The cells were labeled with [^{3}H]TdR for 24 h, autoradiographed, and each colony counted. *Asterisks* indicate the values for the number of viable cells attached which differ significantly ($P < 0.05$) from the mean.

Collagen IV, a basement membrane constituent (3) might theoretically be a better substrate for colonic cells than collagen I. An optimum for collagen IV of 5 to 10 μg/dish was observed with significantly (t test, $P < 0.05$) decreased plating at 1 μg/dish and 50 μg/dish (Fig. 4). Similar data were observed using the high-serum medium (not shown). Decreased [^{3}H]TdR-labeling indexes at 50 and 100 μg/dish ($P < 0.05$) were also seen, indicating that both initial plating and subsequent growth of colonic epithelial cells were inhibited by concentrations of collagen IV 50 μg or greater/35-mm dish.

Fibronectin concentrations of 1 to 100 μg were plated on dried films of 10 μg of collagen IV, the optimal collagen IV concentration (Fig. 3). A peak at 5 μg of FN on the 10-μg collagen IV film was observed ($P < 0.5$ for values at 5, 25, 50, and 100 μg FN different than the mean). No statistical difference in [^{3}H]TdR-labeling indexes which were about 10% in each case, was observed on any of the collage IV-FN mixtures tested. Therefore, DNA replication of the attached cells was not inhibited or stimulated by any of the mixed FN collagen IV substrates tested, as with FN alone.

Cell attachment and growth was then compared on the optimal 10 μg collagen IV-5 μg FN substrate with the 10 μg FN-30 μg collagen I-10 μg BSA mixture. A similar number of cells, 2409 and 2140, respectively, attached to each

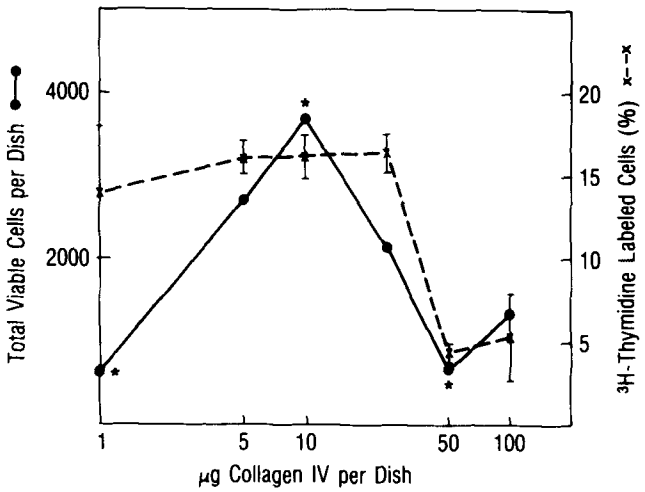

FIG. 4. Inhibition of both attachment and DNA replication in colonic epithelial cells by high concentrations of collagen IV. One milligram of collagen IV was dissolved in 1 ml of 0.1 *M* acetic acid. Appropriate dilutions were added to 1 ml of sterile water in 35-mm petri dishes, and allowed to evaporate to dryness in a laminar flow hood. Each dish was wetted with 1 ml growth medium and the excess removed before addition of equal aliquots of a digested biopsy. Cells were labeled 24 h with [^{3}H]TdR, autoradiographed, and all colonies counted. The mean [^{3}H]TdR-labeling indexes are shown with *error bars* marking the SE. Values for attached cells significantly ($P<0.05$) different from the mean are indicated by *asterisks*.

substrate, and the attached cells had similar [^{3}H]TdR-labeling indexes 10.8 ± 1.1% and 11.1 ± 1.1%. The expensive and tedious preparation of FN over a dried collagen IV base was no better than the cheap simple combination of FN with collagen I and BSA.

Test of Basement Membranes

Basement membrane preparations were screened for colonic cell attachment and growth. Preparations were made from a rat liver epithelial cell line (ARL-18), a bovine capillary endothelial cell strain (BACE), a bovine pulmonary artery endothelial cell line (CPA), and a rat liver endothelial cell line (RLC-4A), (11). Each cell type was seeded at approximately half the confluent cell density into 35-mm petri dishes and treated with 50 μg/ml of ascorbic acid added fresh daily. Cells became confluent by Day 3 and were maintained at confluence for 1 wk. Then the monolayers were washed with PBS, and the cells removed with 0.025 *M* NH_4OH leaving the extracellular matrix (5). None of these basement membrane preparations was markedly better than the others in preliminary experiments, so the choice was made to use the RLC line which readily maintained a contact-inhibited monolayer at confluence for several days.

The extracellular matrix secreted by RLC cells 1 to 21 d after plating was tested for its ability to support attachment and growth of epithelial cells (Fig. 5). The amount of collagen precipitated by the RLC cells on the petri dish as part of the insoluble basement membrane preparation was measured using incorporation of [^{3}H]proline. This value continued to increase after the RLC cells reached confluence (*C* on figure). About 30%of the incorporated [^{3}H]proline was solubilized by collagenase IV digestion, indicating that the incorporation of this hydroxyproline precursor was a measure of collagen deposition. More collagen was secreted into an insoluble basement membrane preparation from 2 to 15 d, and then the incorporation of [^{3}H]proline decreased. Twenty-four days after plating the basement membrane detached from the plate.

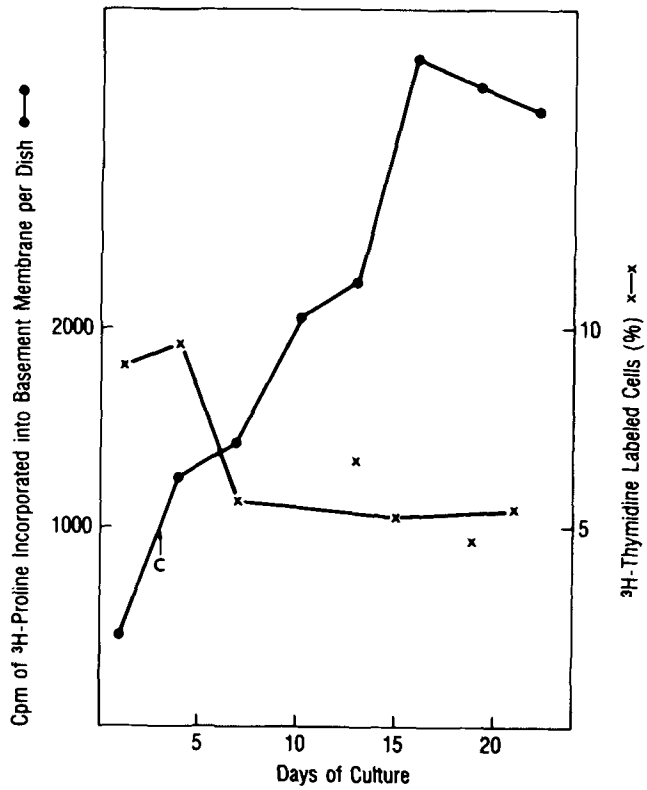

FIG. 5. Measurement of [^{3}H]proline incorporation into rat liver epithelial cell basement membrane and [^{3}H]TdR-labeling index of the attached colonic epithelial cells. RLC cells were plated at approximately 0.5× saturation density in parallel 35-mm plates, and collagen secretion from the cells stimulated by ascorbic acid addition (Methods). *C* indicates the time after plating that the cells become confluent. Collagen deposition into a basement membrane was monitored by the addition of 1 uCi of [^{3}H]L-proline (142 Ci/mmol). The label was added at the timepoints marked by *solid circles* on the figure and the labeling continued for 24 h. The cellular layer was then removed with 0.025 *M* NH_4OH and washed with PBS. The basement membrane remaining attached to the dish was solubilized with 1 ml of 2 *M* NaOH/dish overnight at 37°, and the radioactivity assayed in a scintillation counter. Two dishes were analyzed per timepoint for incorporation of [^{3}H]proline into basement membrane. Variability of incorporation into parallel plates was less than 10%. The mean values are shown as *solid circles*. Two additional dishes were taken at the same timepoints and basement membrane preparations made without [^{3}H]proline labeling. The dried dishes were stored at 4° C until the plating assay. A colonic biopsy digest was plated on the dishes, and the cells labeled with [^{3}H]TdR and autoradiographed (Methods). x = Mean values. The mean of the SEs of the incoporation values was 1.8%, indicating a small spread of the data.

The basement membranes secreted until Day 1, 4, 7, 13, 16, 19, or 21 were tested for their ability to serve as substrates for colonic epithelial cells. Dishes were collected at each time point and stored dry at 4° C until used as a substrate for one biopsy. Two to three times as many cells plated on the basement membrane isolated 1 d after plating compared to the other time points (not shown). Colonic cells plated on the thicker basement membranes secreted for 13 to 19 d displayed less growth than cells plated on basement membranes secreted for 1 to 7 d, as shown by a decrease in [^{3}H]TdR-labeling indexes (Fig. 5). Therefore, as measured by cell attachment and subsequent cell division, the thinner basement membranes were the best.

Comparison of Substrates

Cell attachment and their subsequent replication were assayed on six different substrates: gelatin, the mixture of FN-collagen I-BSA, collagen I plus only BSA, collagen I alone, collagen IV alone, and a basement membrane preparation secreted by the rat liver endothelial cell line, RLC, after 1 wk at confluence (optimal time for DNA replication). Data showing the mean number of colonic epithelial cells cultured from eight different colonic biopsies are summarized in Table 4. In some experiments, such as #148 which is also shown in Table 3, additional substrates were tested and not indicated in Table 4 because they were not tested using other biopsies. Eight biopsies were tested to control for possible interindividual variation. Colonic biopsies are taken from patients using a biopsy forceps, which is not a precise instrument but gives a relatively similar-sized biopsy. Because of this inherent size variability between specimens and uncontrollable differences in their viability, all the data were standardized to one substrate—the mixture of collagen I, FN, and BSA and then the mean and SE of the summed experimental data were calculated (Table 4). Thus the mean number of cells cultured per dish, the mean number of colonies per dish, the average size of the colonies per dish, and the mean [^{3}H]TdR-labeling indexes per dish were summed over the eight biopsies and expressed as ratios to 1, the value given to the substrate using a mxiture of collagen I, FN, and BSA.

Collagen I alone, diluted into medium and allowed to attach for 2 h in the incubator before use, gave statistically higher numbers of cultured cells, more colonies per dish, and larger colonies than the collagen I-FN-BSA substrate. Collagen I plus BSA also gave the highest numbers in each category, but because of the variability between the biopsies tested the increase was marginally significant with $0.05 < P < 0.1$. Collagen I alone or with BSA or BSA and FN gave identical [^{3}H]TdR-labeling indexes. Therefore, the effect of addition of BSA or BSA plus FN to collagen I was only on cell attachment, not on subsequent cell growth.

Plating on gelatin films gave fewer attached cells, fewer colonies, and reduced proliferation in those colonies. Plating on the basement membrane preparation and on collagen IV gave fewer cultured cells, smaller colonies, statistically fewer colonies and in the case of collagen IV, decreased proliferation of the plated cells.

The collagen I substrate was the best of those tested. The addition of BSA resulted in more attached cells and more colonies in the three biopsies tested. The addition of BSA was simple and not detrimental to cell attachment and growth, so further experiments were performed on collagen I-BSA substrates. The frequency of colony formation from biopsy digests was assayed with 26 additional biopsies to those described in Table 4. A total of 97% (28 of 29) biopsies yielded cultured colonic epithelial cells, demonstrating that a high success rate for patient screening was achievable using this substrate with serum-free medium.

DISCUSSION

Development of a new protocol for the routine tissue culture of epithelial cells from colonic biopsies has been

TABLE 4

MEAN NUMBER OF COLONIC EPITHELIAL CELLS CULTURED PER DISH ON DIFFERENT TEST SUBSTRATES[a]

Experiment No.	Gelatin	Collagen I + BSA + FN	Collagen I + BSA	Collagen I	Collagen IV	RLC BstMb
159	0	1656	3094	1621	507	410
148	-	2398	10 584	4887	-	-
158	0	658	809	645	0	273
119	673	6465	-	-	4208	1234
121	393	2198	-	-	3711	-
145	0	6728	-	9449	-	-
153	-	5544	-	-	-	397
154	-	666	-	1322	-	-
Relative Number of Cells Cultured per Dish, Mean (SE)						
	0.06[b] (0.04)	1	2.50 (.98)	1.48[c] (0.24)	0.66 (0.37)	0.23[b] (0.07)
Relative Number of Colonies Cultured per Dish, Mean (SE)						
	0.01[b] (0.01)	1	1.8 (0.6)	1.2[c] (0.1)	0.4[b] (0.1)	0.2[b] (.06)
Relative Colony Size per Dish, Mean (SE)						
	0.50 (0.29)	1	1.38 (0.16)	1.24[c] (0.11)	0.82[b] (0.04)	0.83 (0.14)
Relative [^{3}H]TdR-Labeling Index per Dish, Mean (SE)						
	0.24[b] (0.19)	1	1.01 (0.11)	1.06 (0.10)	0.85[b] (0.04)	0.83 (0.11)

[a] Plated cells were labeled for 24 h with [^{3}H]TdR, fixed, and counted after autoradiography (Methods). Because the size and viability of the biopsy could not be completely controlled, all results were standarized to the values obtained using the collagen I-FN-BSA substrate, and then the mean relative values for each substrate were compared to the standard value of 1 using the one-tailed t test.

[b] Indicates values lower than the mean with $P < 0.05$. The substrates tested were a 0.1% gelatin film (remaining after application of 1 ml/dish), a mixture of 30 μg collagen I, 10 μg BSA, and 10 μg FN/ml per dish; a mixture of 30 μg collagen I and 10 μg BSA per ml per dish; 30 μg collagen I; 10 μg collagen IV dried to the 35-mm dish according to the supplier's specifications; RLC BstMb, a basement membrane preparation made from NH_4OH-treated RLC cell cultures confluent for 1 wk. A mean of eight dishes was plated from each digested biopsy.

[c] Indicates those values higher than the mean, with $P < 0.05$.

achieved using a serum-free medium and a nongelled collagen I-BSA substrate (protocol 2). This substrate was superior to basement membrane preparations from endothelial cells, mixtures of collagen I or IV with FN, and gelled collagen I. Ninety-seven percent of a series of 29 biopsies could be cultured by this new protocol 2, not significantly higher than the 91% yield obtained in our earlier study with the high serum-gelatin substrate method (protocol 1, 7). However, about four times as many cells were cultured from each biopsy using protocol 2 compared with protocol 1.

Analysis of biopsies taken over a period of time allows the physiologic state of colonic cells in individual patients to be monitored. Of particular interest to this laboratory are patients at high risk to develop colon cancer because of their development of benign tumors or their family history of colon cancer. These patients exhibit abnormal, hyperproliferating colonic cells (7). Colonic cells from such high-risk patients showed a decrease of their characteristic hyperproliferation to normal growth rates when cultured by protocol 2 in media supplemented with $CaCl_2$ concentrations above the optimum for growth, 0.1 m*M* (2). Growth inhibition of colonic cells in vivo was also seen in patients on a diet supplemented with high levels of calcium. Ingestion of high levels of calcium would leave high Ca^{++} levels in the colonic lumenal contents because most would not be absorbed (2).

Biopsies are processed in the laboratory within minutes of removal from the patient. This is an important consideration in working with colon tissue which rapidly autolyzes. Other investigators have reported good growth in suspension culture of normal colonic epithelial cells from surgical resections (16). However, in our experience normal colonic epithelial cells could be cultured only rarely from resected specimens. These surgical specimens are received in the lab after review in Surgical Pathology, and hours after the blood supply has been cut off. Much of the tissue dies during this long period between its removal from the patient in the surgical suite and its arrival in our laboratory.

Excellent growth of human fetal colonic epithelial cells has been reported (17) without the extensive modifications of medium, digestion conditions, and growth conditions discussed in this paper and others from this laboratory on colonic epithelial cells from adults. We found in earlier studies that fetal colon was as easy to primary culture as adenomas or carcinomas, and was much hardier than adult normal colonic epithelial cells.

The medium chosen for these studies was the supplemented NCTC 168 used in earlier studies for normal epithelial cell growth (7) with the $CaCl_2$ level reduced to the optimum for growth, 0.1 m*M*, and without fetal bovine serum. Inhibition of the growth of normal epithelial cells by serum has been reported for a variety of epithelial cell types including human keratinocytes (20), human mammary epithelial cells (10), adult rat hepatocytes (4), and human bronchial epithelial cells (13). Transforming growth factor beta (TGFβ), a factor released from platelets, has recently been shown to arrest normal human prokeratinocytes in the G1 phase of the cell cycle (15) and to induce differentiation of human bronchial epithelial cells (14). TGFβ may be one of the growth-inhibitory factors found in serum. Fetuin and high density lipoprotein, also found in serum, inhibited the colony-forming efficiency of mouse keratinocytes (1). Ethanolamine and phosphoethanolamine have been shown to improve the serum-free growth of keratinocytes and mammary epithelium (6,10,20), and so were added to our new medium formulation.

The new digestion protocol includes the use of acetylcysteine to remove some of the mucous secreted by goblet cells in the colon. Bacteria from the septic colon adhere to the mucous strands and often lead to contamination on cultures held more than 48 h. Reduction of the amount of mucous left on the plated cells reduced the amount of contamination.

Basement membrane preparations from endothelial cells are in theory a more natural substrate for epithelial cells than purified substrates. Basement membrane preparations from four different cell lines proved disappointing, possibly because colonic cell attachment was inhibited by high concentrations of collagen I and collagen IV and gelled collagen I. The basement membrane preparations became worse substrates as more collagen was deposited into extracellular matrix over time. The basement membrane constituent FN enhanced cell migration (data not shown), but did not improve, and sometimes impaired, cell attachment when added to collagen I and BSA. The basement membrane constituent laminin, dried to dishes using the supplier's specifications (Bethesda Research Laboratories), was tested initially in place of gelatin. So few colonic cells attached to laminin that no further work was done with it.

The best substrate for adult human colonic epithelial cells was a simple mixture of commercially available, ungelled collagen I and BSA, diluted into additive-free, calcium-free NCTC 168. The physical state of the collagen substrate is quite important. Gelled collagen I was a very poor substrate for colon cells in this study. In the absence of fetal bovine serum, air-dried collagen I was a better substrate for adult rat hepatocytes than saline-precipitated collagen and FN (12). Collagen IV was not required for adult rat hepatocyte growth (12) or adult human colonic epithelial cell growth in culture. Biomatrix preparations from colon tissue were not tested for the attachment and growth of normal colon cells because biomatrix preparations did not improve the plating or growth of benign colon tumor cells in an earlier study (8). Adult colon epithelial cells cultured in serum-free medium still have a short life of no more than 4 d. The new protocol for colonic biopsy digestion and plating using serum-free medium is a starting point for the investigation of growth modulators of normal colon epithelial cells.

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