

Actin Cytoskeletal Organization Loss in the Benign-to-Malignant Tumor Transition in Cultured Human Colonic Epithelial Cells

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ABSTRACT

The colonic epithelium *in vivo* is a highly indented sheet one cell thick. Culture methods have been developed to allow the normal cellular migration of the cells comprising this sheet to flatten it into a patch on the surface of a Petri dish [Friedman, E. A., Higgins, P. J., Lipkin, M., Shinya, H., and Gelb, A. M., *In Vitro* (Rockville), 17: 632-644, 1981]. Actin cytoskeletal organization was analyzed in such epithelial "patches" derived from several human colonic adenocarcinomas and their precursors, adenomas (benign tumors). The actin cytoskeleton was visualized by fluorescence microscopy after the fixed, permeabilized cells were stained with rhodamine-conjugated phalloidin. This drug has a very high affinity for actin filaments and a much lower affinity for monomeric actin. Actin organization was scored from 0 (no cables) to 5 points (extensive intercellular cable network). The phalloidin-stained actin found in seven adenocarcinomas had a predominantly granular fluorescence pattern with very little cable organization, scoring an average of 0.9 ± 0.8 (S. D.). Three established cell lines derived from human colon carcinomas contained no cables by this analysis, scoring 0.0 ± 0.0 . In marked contrast, all 12 of the cultured adenomas had extensive actin cable networks, scoring an average of 4.3 ± 0.4 . There was no statistical difference between adenomas of differing histopathology class and malignant potential. However, cytoskeletons of plasminogen-activator-secreting "late-stage" preneoplastic cells from adenomas became disorganized by exposure to 12-O-tetradecanoyl-phorbol-13-acetate or another tumor promoter, teleocidin B. They scored, respectively, average actin organization values of 0.0 ± 0.0 and 0.4 ± 0.6 . In contrast, nonplasminogen-activator-secreting "early-stage" preneoplastic cells from less advanced benign tumors were unaffected by 12-O-tetradecanoyl-phorbol-13-acetate or teleocidin B and retained extensive actin organization. Most, if not all, adenocarcinomas arise from preexisting preneoplastic adenomatous cells. Thus, loss of actin organization appears to mark the transition of noninvasive benign colonic tumors to invasive malignant tumors in humans. This transition is mimicked *in vitro* by exposure of certain "late-stage" preneoplastic cells to a tumor promoter which induces secretion of a plasminogen activator.

INTRODUCTION

A flexible actin cytoskeleton characterizes eukaryotic nonmuscle cells. Polymerization and depolymerization of actin filaments enables cell shape to change during mitosis and cell movement. In many instances, the normal balance between these 2 proc-

esses is perturbed by transformation of the cell, so that most actin bundles are lost. Immunofluorescence techniques have shown that the distinct actin bundles characterizing the untransformed fibroblast are replaced by a diffuse intracellular fluorescence (6, 23). Since little, if any, change in the concentration of actin per cell has been reported to accompany transformation of fibroblasts (12, 26), it has been concluded that transformation prevents cellular actin from forming the thick bundles visualized by light microscopy techniques. Perhaps the recycling actin monomers are shifted into networks under the plasma membrane.

Using fibroblast transformation as our model, we assumed that the actin cytoskeleton would become disorganized at a stage in human colon carcinoma development when tumors are first seen, the benign tumor stage. Benign tumors or adenomas are the direct antecedents of colon carcinomas in humans (14, 20). However, preneoplastic stages are known to occur in colonic epithelium prior to the appearance of benign tumors (4). These are indicated in Chart 1 as alterations characteristic of hereditary colon cancer syndromes. Previous studies had shown that the actin cytoskeleton was somewhat disordered in the dermal fibroblasts of patients with the hereditary colon cancer syndrome familial polyposis compared to cells from normal individuals (15). A similar loss of cytoskeletal organization was expected in the colonic epithelium of these patients, and a greater loss was expected in the epithelial cells of benign tumors. It was therefore a surprise to find a highly organized actin cytoskeleton in epithelial cells from adenomas in a preliminary experiment. These benign tumors contain no areas of normal epithelial cells. All of the cells in the adenoma crypts look similar, relatively undifferentiated with little mucus and with abnormal-appearing nuclei. Therefore, we were assaying premalignant cells, not normal cells.

Benign tumors can grow to several cm in diameter, and sometimes these large tumors are indistinguishable by eye from carcinomas. However, the adenomas are never invasive and always protrude into the gut lumen, not down into the muscle and fat layers surrounding the epithelial layer as carcinomas do. Adenoma cells have lost growth control, forming colonic crypts several times normal length. Similar changes are seen in carcinomas, but they occur first in adenomas and do not characterize earlier preneoplastic stages (Chart 1).

Adenomas can be composed of several different types of preneoplastic as well as neoplastic cells. The histological class of villous adenomas which display serrated crypts in cross-section (Fig. 1b) give rise to carcinoma cells with high frequency (41%), as shown by examination of sectioned tumors (20). The histologically distinguishable tubular adenomas generally do not contain carcinoma (20). They have oval crypts in cross-section (Fig. 1a). The mixed class of villotubular adenomas contains carcinoma cells at an intermediate rate of 22% (20). The actual premalignant cell within these classes is the dysplastic cell (14)

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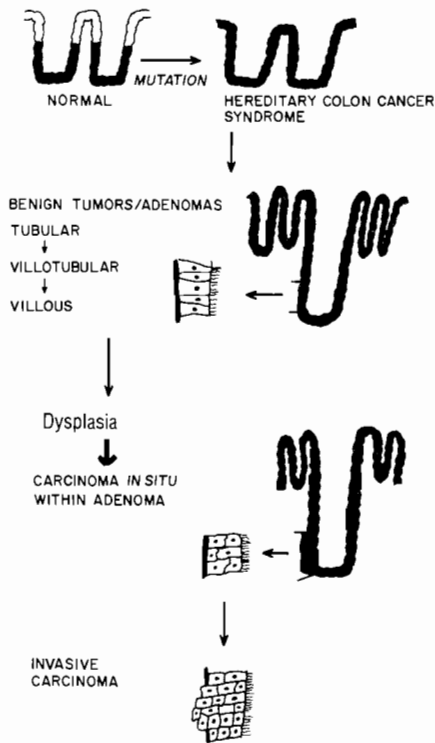


Chart 1. Model for human colon carcinoma evolution through a series of preneoplastic stages (adapted from Ref. 8). The drawings depict longitudinal sections through the colonic mucosa showing the 1-cell epithelial layer in outline. Individual cells are not shown, but the area of cells capable of DNA replication is *blackened*, while the area of nonreplicating, terminally differentiated cells at the top of the normal colonic crypt is left *white*. The earliest preneoplastic changes in the development of colon cancer in genetically normal individuals are believed to be the same ones which occur with great frequency in the inherited colon cancer syndromes such as familial polyposis. The change is simple. Cells continue to replicate as they migrate up the crypt instead of ceasing replication in concert with normal epithelial differentiation after 2 or 3 divisions. This slight hyperplasia is caused by a genetic change, as the disorder is inherited as a dominant phenotype in about 50% of the progeny of an affected individual. The stem cell in these inherited disorders must be altered, as the change in proliferation pattern characterizes the entire crypt. In a rare inherited disorder like familial polyposis, the overwhelming majority of colonic crypts are abnormal. This aberrant and inherited pattern of DNA replication must be caused by an altered preneoplastic oncogene.

One or more further genetic changes then must occur for an adenoma to form. Abnormally proliferating cells characterize the adenoma, as well as the earlier stages seen in familial colon cancer syndromes. In the adenoma, however, these cells do not exfoliate normally. The crypts become elongated. The adenoma cells are noticeably less differentiated than are normal cells and are more abnormal in appearance. Two histologically distinct adenomas (Fig. 1) are seen with differing malignant potential. Tubular adenomas infrequently give rise to a carcinoma. In a tubular adenoma, cross-sections of the colonic crypts look like tubes. In a villous adenoma, cross-sections of the colonic crypts look like serrated leaves or palm fronds. The cells from the villous adenomas are more aberrant in appearance than are the tubular cells and much more likely to progress to malignancy. Because of this and their clinical prognosis, villous cells are considered to be at a later preneoplastic stage.

In focal areas within adenomas, morphologically aberrant dysplastic cells arise which may be multilayered or, at least, pseudostratified (see Fig. 1c). Carcinoma arising within an adenoma exhibits epithelial layers more than 1 cell thick as neighboring crypts adhere (see Fig. 1d). At the final stage, the adenocarcinoma migrates through the muscular wall surrounding the epithelial layer (see Fig. 1e).

(Fig. 1c), which, in turn, gives rise to carcinoma *in situ* (Fig. 1d). When the carcinoma becomes invasive, it is termed infiltrating (Fig. 1e). We decided to test adenomas of different histological classes to see whether the extensive actin cytoskeleton was a consistent feature of benign tumors, to test whether the presence of dysplastic cells or carcinoma within the adenoma disrupted the actin cytoskeleton, and to compare these results with those characterizing metastatic and nonmetastatic colonic carcinomas under the same conditions of primary culture.

MATERIALS AND METHODS

Tissue Culture of Tumors. The procedure is essentially as described (7). Tumor segments were washed 3 times in antibiotic-containing wash medium, and then the viable carcinoma cells were dissected from any necrotic tissue, fibrous growth, or fat. Adenoma segments were taken from the head of the polyp at removal from the patient so that additional dissection was unnecessary. The tissue was then minced into approximately 1-cm pieces and digested in hyaluronidase (300 units/ml; Type VI, Sigma Chemical Co.), collagenase (600 units/ml; type IV, Worthington), and neuraminidase (4 units/ml; type V, Sigma). The tissue was digested in a mixture consisting of equal 1-, 2-, 3-, 4-, or 5-ml aliquots of each enzyme plus wash medium, depending on the size of the packed volume of minced, pelleted tumor (~1 to 5 ml). The digested tumor consisted of a mixture of small groups of attached epithelial cells, partial or complete colonic crypts, and T- and B-lymphocytes, RBC, and any infiltrating leukocytes freed from the intercryptal lamina propria. The majority of the latter cells were removed by differential centrifugation. The epithelial digests were then plated in 0.1 ml of NCTC 168 medium per plate (K. C. Biologicals, Lenexa, KS) in 20 to 30 gelatin-coated 35-mm dishes, depending on the size of the tumor. After 30 min at 37° in the incubator to allow for attachment, 1 ml of 37° medium was added carefully to the plates, which were left undisturbed overnight. Colonies were fixed for actin analysis after primary (see Table 1) culture directly on the tissue culture plate. They were not transferred to glass coverslips. Medium NCTC 168 was supplemented with fetal calf serum, transferrin, insulin, hydrocortisone, epidermal growth factor, and selenous acid. Deoxycholic acid at 8×10^{-9} and pentagastrin at 250 $\mu\text{g/ml}$ were also added. After an overnight incubation, cells in attached crypt fragments were observed to have migrated onto the plastic Petri dish to form a flat patch (Fig. 2).

Materials. TPA³ and 4 α -PDD were purchased from P. L. Biochemicals, Milwaukee, WI. Stocks at 10 $\mu\text{g/ml}$ in dimethyl sulfoxide were kept at -20°, desiccated, and shielded from light. Working solutions were made up just before addition to cultures. Teleocidin B was the kind gift of Dr. Takashi Sugimura, National Cancer Center of Japan, was stored at 10 $\mu\text{g/ml}$ in dimethyl sulfoxide, and was used under the same conditions as was TPA (28). DOC was purchased from Aldrich Chemical Co. (Milwaukee, WI), dissolved in ethanol at 10 mM, diluted to 10^{-5} M in PBS, filter-sterilized, and stored at 4°.

Tumor Procurement. Individual adenomas were removed by colonoscopy. A portion of the side of the head of the adenoma was removed and placed immediately into cold wash medium for transport to the laboratory. The stalk and tip of the adenoma were never sampled because of their importance in diagnosis. The adenomas selected were always at least 1 cm or more in diameter, as sampling of smaller adenomas might compromise their diagnosis. Portions of carcinoma were received from Surgical Pathology. All tumors were number-coded to protect patient confidentiality.

Actin Cytoskeleton Visualization. The medium from a culture was carefully removed and replaced with 3.8% (v/v) formaldehyde in PBS. The culture was stored at 4° covered with parafilm until assay. The cultures were identified by sequential numbering and not by the histopathology of the specimen, which, in all cases, was unknown to the investigators until the completion of the experiment because of the time necessary to prepare the pathology report. The fixed colonies were washed twice with PBS, permeabilized with 1% Nonidet P-40 for 2 min, washed twice with PBS, stained with rhodamine-coupled phalloidin (4 $\mu\text{g/ml}$) at 37° for 30 min, and then washed twice again with PBS, and coverslips were mounted over the colonies with Aquamount (29). Random areas of each colony were photographed using Tri-X (Kodak) black-and-white film. An average of 4 photographs were taken of each culture. Duplicate photographs were printed to enable each group to have a set for independent scoring.

³ The abbreviations used are: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; 4 α -PDD, 4 α -phorbol-13-decanoate; PA, plasminogen activator; DOC, deoxycholic acid; PBS, phosphate-buffered saline.

Quantitation of Actin Organization. Two photographs were printed (4 x 5") from each frame and labeled on the back with the experiment number, and treatment of the cells, if any, was performed. Two of the investigators (E. F. and M. V.) each held a set of these photographs. At the conclusion of the experimental work, all of the photographs were examined independently, and 6 classes of actin-cytoskeletal organization became apparent. They were given an arbitrary numerical value from 0 to 5 as described below. Two investigators (E. F. and M. V.) at separate locations agreed verbally on the categories and, independently and without knowledge of the identification of the photographs, which were number-coded, they classified each photograph. The results of each analysis were independently tabulated, averaged, and compared as discussed in the text.

5 Points. The actin network connecting the cells into a tissue is more prominent than are the individual cellular outlines. Actin cables are distinct, and they span more than one cell, running into focal areas between cells (Fig. 3, a and b).

4 Points. Actin cables appear at the periphery more distinctly than within the cells, giving an obvious outline to each cell in the intact monolayer. No network is obvious, as was seen in Class 5. The cell boundaries are angular and not round (Fig. 3, c and d).

3 Points. Cells grow as a tightly packed monolayer as in Classes 4 and 5. Some cables are visible within cells, but individual cells are apparent instead of a network. Cell peripheries are not especially strongly staining in contrast to Class 4 (Fig. 3e).

2 Points. Cells grow as an interrupted monolayer, which looks quite thick. Actin cables within cells are sometimes seen. The cells often end in "lily-pad" formations (Fig. 3f).

1 Point. Cells grow in a thin, interrupted monolayer with granular actin and no intact cables (Fig. 3, g and h).

0 Point. Cells are round with granular actin concentrated around the periphery of the cells. Sometimes cells form a monolayer, but their

boundaries are rounded and not angular as in Classes 3 and 4 (Fig. 3, i and j) and do not contain actin bundles.

RESULTS

Adenocarcinomas with Little Actin Cytoskeletal Organization. The actin cytoskeletal organization was assayed, as described in "Materials and Methods." Six adenocarcinomas in primary culture 1 to 3 days after plating, one adenocarcinoma 6 days postplating (Table 1), and 3 colon carcinoma established cell lines (HT-29, SW480, SW1116) were examined. Four of the adenocarcinomas had metastasized to the regional lymph nodes and were classified as Dukes' C (5). The remaining 3 had not metastasized and were classified as either Dukes' A or Dukes' B, depending on the extent of their penetration into the muscular wall and pericolonic fat. All of the photographs of each cell line (an example is shown in Fig. 3j) were placed in the "0" class by each investigator, scoring 0.0, with a combined standard deviation of 0.0. (Table 1). The metastatic adenocarcinomas scored an average of 0.7 ± 0.5 (S. D.), while the nonmetastatic ones scored 1.0 ± 1.0 (Table 2). There were no statistical differences in actin organization between the metastatic and nonmetastatic adenocarcinomas or the carcinomas in primary culture taken as a group, compared with the established cell lines by Student's *t* test (Table 3). The adenocarcinoma groupings that were apparent were those scoring 0.0 to 0.2 (692, 586, and 711, shown in Fig. 3i), those scoring 0.9 and 1.0 (700 and 716), and those scoring 1.8 and 2.0 (574, shown in Fig. 3h, and 717) (Table 1). The significance, if any, of these groupings is not clear at present.

Table 1
Actin organization scores for preneoplastic and malignant cells

	Days in culture	Scorer 1	Scorer 2	Combined	Pathology	Morphological changes induced by TPA
Adenomas						
Tubular						
616	3	4.5 ± 1.2^{ab}	3.8 ± 1.8	4.2 ± 1.5	No dysplasia	-
690	3	5.0 ± 0.0	3.3 ± 1.6	4.0 ± 1.5	No dysplasia	-
Villotubular						
572	1	4.9 ± 0.3	5.0 ± 0.0	4.9 ± 0.3	Carcinoma <i>in situ</i> in head	+
588	3	3.7 ± 1.2	3.3 ± 1.5	3.5 ± 1.2	Severe dysplasia	+
596	4	5.0 ± 0.0	3.7 ± 0.6	4.5 ± 0.8	No dysplasia	-
668	5	4.8 ± 0.4	3.4 ± 1.6	4.1 ± 1.4	No dysplasia	-
674	4	4.8 ± 0.5	4.0 ± 0.7	4.4 ± 0.7	Moderate dysplasia	+/- ^c
Villous						
558	1	4.6 ± 0.5	2.8 ± 0.8	3.9 ± 1.1	Infiltrating carcinoma	+
676	4	5.0 ± 0.0	4.5 ± 0.7	4.8 ± 0.5	Moderate dysplasia	+
678	4	5.0 ± 0.0	4.5 ± 0.7	4.8 ± 0.5	No dysplasia	-
708	4	4.5 ± 0.8	4.8 ± 0.4	4.7 ± 0.7	Infiltrating carcinoma	+
713	4	4.0 ± 1.7	4.3 ± 1.2	4.2 ± 1.3	Moderate dysplasia	+
Carcinomas						
Nonmetastatic						
574	1	1.6 ± 1.7	2.2 ± 1.9	1.8 ± 1.8	Dukes' B	
586	2	0.0 ± 0.0	0.3 ± 0.6	0.2 ± 0.5	Dukes' B	
711	1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	Dukes' B	
717	1	1.0 ± 0.0	3.0 ± 0.0	2.0 ± 1.2	Dukes' A	
Metastatic						
692	3	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.3	Dukes' C	
700	6	0 ^d	1.5 ± 2.1	1.0 ± 1.7	Dukes' C	
716	3	0.5 ± 0.6	1.3 ± 1.9	0.9 ± 1.4	Dukes' C	
Cell lines						
HT-29		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		
SW480		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		
SW1116		0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		

^a Mean \pm S.D.

^b The overall average was computed by adding scores for all photographs in both sets and dividing by the total number of photographs.

^c Some epithelial patches were morphologically clustered; others were not.

^d Only 1 photograph was scored.

Table 2
Summary of actin scores

Cells	Score
Adenoma	4.3 ± 0.4 ^a
Tubular	4.1 ± 0.1
Villotubular	4.3 ± 0.5
Villous	4.5 ± 0.4
Carcinoma	0.9 ± 0.8
Nonmetastatic	1.0 ± 1.0
Metastatic	0.7 ± 0.5
Carcinoma cell lines	0.0 ± 0.0

^a Mean ± S.D.

Adenomas with Extensive Actin Cytoskeletal Organization.

Two tubular, 5 villotubular, and 5 villous adenomas, all benign tumors, were maintained in primary culture 1 to 5 days before fixation. All of the adenomas had highly organized actin cytoskeletons (see Fig. 3, a to e). Their actin organization scores averaged 4.3 ± 0.4 , varying only between 3.5 to 4.9 (Table 1). The investigators at both institutions independently scored each adenoma quite similarly, yielding a combined standard deviation of only 0.4. The 12 benign tumors were therefore markedly different in their cytoskeletal organization than either the 7 adenocarcinomas in primary culture or the 3 carcinoma cell lines, which averaged 0.9 ± 0.8 and 0.0 ± 0.0 , respectively. The differences between adenomas and carcinomas in primary culture or lines are statistically significant, with $p < 0.001$ for both comparisons (Table 3). There was no evidence for a stepwise loss of actin organization between adenoma classes, as the tubular, villotubular, and villous adenomas scored 4.1 ± 0.1 , 4.3 ± 0.5 , and 4.5 ± 0.4 , respectively, with no statistical differences between the values (Tables 2 and 3).

Disruption of Actin Cytoskeleton by PA Secreted in Response to Promoting Agents.

When preneoplastic cells from adenomas were placed in primary culture and exposed to TPA, they displayed 1 of 2 phenotypic responses. If the adenomas contained areas of infiltrating carcinomas or contained "late-stage" premalignant cells, these cells secreted a PA. In response to this protease, the secreting cells and those around them first elongated. Then some detached from the Petri dish but remained attached to adjacent cells. This resulted in the formation of multicellular clusters. These advanced premalignant cells include moderately and severely dysplastic cells and at least one class of villous cells (Refs. 7 and 11; see "Introduction" and Fig. 1 for histopathology classes). Cultured pure tubular adenomas secrete no PA in response to TPA, but they exhibit a mild proliferative response. This tubular class is considered to be at an earlier preneoplastic stage than is the villous class, as the tubular adenomas infrequently contain foci of carcinoma (20) and are generally smaller in size. The mixed adenoma class, the villotubular, displays either the tubular response, the villous clustering response, or both responses in different epithelial patches, depending on the portion of the tumor taken for culture. The TPA-induced PA secretion characteristics of premalignant cells in these adenomas are maintained by malignant cells. Each of 5 cultured adenocarcinomas secreted PA in response to TPA and became altered morphologically (7).

Each of the 12 adenoma monolayers cultured in this study was treated with TPA (50 ng/ml) for 24 hr, and its response was observed. Neither tubular adenoma contained any more advanced premalignant cell types, but were pure tubular and thus

unambiguously early-stage preneoplastic. They exhibited no morphological response to TPA (Table 1). Three of the villotubular adenomas, 572, 596 and 668, also were unchanged in appearance after exposure to TPA. Two contained no dysplastic cells, and the third had carcinoma *in situ* within the head of the polyp, which was never sampled for culture. Thus, the adenomatous cells cultured were probably pure tubular cells in each case. The other 2 villotubular adenomas, 588 and 674, contained dysplastic cells and were morphologically altered by TPA. Four of the 5 villous adenomas contained either dysplastic cells or carcinoma, and each secreted PA in response to TPA. The sole unresponsive villous adenoma had no areas of dysplasia. This is the only non-TPA-responsive villous adenoma we have seen in the 14 thus far studied in this laboratory. This result demonstrates heterogeneity within villous adenomas with some but not all villous cells capable of PA secretion (11).

The effects of TPA on actin cytoskeletal organization were analyzed in 3 of the TPA-unresponsive adenomas (596, 690, and 668). Parallel primary cultures from each adenoma were exposed for 24 hr to promoting agents—TPA (50 ng/ml), 4 α -PDD (50 ng/ml), (an inactive analogue), teleocidin B (50 ng/ml), or 10^{-7} M of the endogenous promoting agent DOC—or were left untreated. Each of the adenomas failed to respond to TPA by morphological changes. They also exhibited no TPA-induced alterations in actin cytoskeleton. The values obtained were not statistically different from the control values or those of the cultures exposed to 10^{-7} M DOC (Table 3). Untreated TPA-unresponsive preneoplastic cells exhibited an average actin score of 4.2 ± 0.3 , and the TPA-treated cultures scored 4.2 ± 0.4 , while the DOC-treated cultures scored 4.0 ± 0.4 . DOC, a secondary bile acid found in the colon, is an endogenous tumor promoter. Increasing its concentration by various means in the intestines of animals pretreated with a

Table 3
Determination of statistical significance of differences in actin scores by *t* tests

	d.f.	<i>t</i>	LS ^a
Types of adenomas			
Tubular (2) ^b vs. villotubular (5)	5	0.46	
Tubular (2) vs. villous (5)	5	0.52	
Villotubular (5) vs. villous (5)	8	0.68	
Types of carcinomas			
Metastatic carcinoma (3) vs. nonmetastatic carcinoma (4)	5	0.50	
Adenomas (12) vs. carcinomas (7)	17	12.3	<0.001
Adenomas (12) vs. cell lines (3)	13	17.1	<0.001
Carcinomas (7) vs. cell lines (3)	8	1.8	
Promoter additions (comparisons only if >1 data point/condition)			
TPA-responder vs. TPA-nonresponder			
Control (3) vs. Control (3)	4	0.08	
DOC (10^{-7} M) (2) vs. DOC (2)	2	0.51	
TPA (50 ng/ml) (2) vs. TPA (3)	3	12.9	0.001
4 α -PDD (50 ng/ml) (2) vs. 4 α -PDD (3)	3	4.50	<0.05
TPA-responsive			
Control (3) vs. TPA (2)	3	8.59	0.01
Control (3) vs. DOC (2)	3	0.76	
Control (3) vs. 4 α -PDD (2)	3	2.37	
Control (3) vs. teleocidin (50 ng/ml) (2)	3	6.62	<0.01
TPA-nonresponders			
Control (3) vs. TPA (3)	4	0	
Control (3) vs. DOC (2)	3	0.67	
Control (3) vs. 4 α -PDD (3)	4	2.8	<0.05
Nonresponder (3) vs. responder (2)	3	21.3	<0.001
Control vs. TPA			
Nonresponder (3) vs. responder (3)	4	0.07	
TPA vs. control			

^a LS, level of significance.

^b Numbers in parentheses, number assayed.

Table 4
Response of adenomas to promoters

	Control	DOC (10 ⁻⁷ M)	TPA (50 ng/ml)	4 α -PDD (50 ng/ml)	Teleocidin (50 ng/ml)
Late-stage preneoplastic and TPA responders					
588	3.5 \pm 1.2 ^a	3.2 \pm 1.0	0.0 \pm 0.0	2.0 \pm 1.1	ND ^b
676	4.8 \pm 0.5	ND	— ^c	3.2 \pm 1.2	0.8 \pm 0.8
713	4.2 \pm 1.3	4.2 \pm 0.8	0.0 \pm 0.0	ND	0.0 \pm 0.0
Early-stage preneoplastic and TPA-nonresponders					
596	4.5 \pm 0.8	3.7 \pm 1.0	4.0 \pm 1.0	4.5 \pm 0.6	ND
690	4.0 \pm 1.5	4.3 \pm 1.0	3.9 \pm 1.4	5.0 \pm 0.0	ND
668	4.1 \pm 1.4	ND	4.7 \pm 0.7	5.0 \pm 0.0	3.8 \pm 1.3
Summary					
TPA-responsive (late preneoplastic)	4.2 \pm 0.7	3.7 \pm 0.7	0.0 \pm 0.0	2.6 \pm 0.8	0.4 \pm 0.6
TPA-nonresponsive (early preneoplastic)	4.2 \pm 0.3	4.0 \pm 0.4	4.2 \pm 0.4	4.8 \pm 0.3	ND

^a Mean \pm S.D.

^b ND, not determined.

^c This condition was lost during actin analysis, but the cells were observed to cluster in the presence of TPA (50 ng/ml).

carcinogen caused an increase in tumor incidence (1, 21). DOC and TPA at the concentrations used here stimulate DNA replication but not PA release in tubular adenomatous cell cultures (7). Neither had any effect on actin organization in the tubular adenoma cultures in this study. Surprisingly, 4 α -PDD, the TPA analogue enhanced actin organization by a small but meaningful amount (note the small standard deviation of the measurements): 4.2 \pm 0.3 to 4.8 \pm 0.3 ($p < .05$; Table 3). Possibly, 4 α -PDD inhibited actin cable depolymerization or reorganization. Cultures of adenoma 668 treated with teleocidin B exhibited neither morphological alterations nor major alterations in actin organization, scoring 3.8 \pm 1.3. Thus, none of the 3 promoting agents had any effect on actin cytoskeletal organization in "early-stage" preneoplastic cells, and none induced PA release.

Three TPA-responsive adenomas were analyzed for the effects of the 3 tumor promoters on actin organization. Parallel primary cultures of villous adenomas 713 and 676 and villotubular adenoma 588 were treated for 24 hr with TPA (50 ng/ml), 4 α -PDD (50 ng/ml), teleocidin B (50 ng/ml), or 10⁻⁷ M DOC or were left untreated. The untreated, 4 α -PDD-treated, and DOC-treated cultures had averaged actin scores of 4.2 \pm 0.7, 2.6 \pm 0.8, and 3.7 \pm 0.7 (Tables 3 and 4). Both TPA and teleocidin B induced cell clustering and concomitant loss of actin cables (Fig. 4). TPA treatment reduced the actin-organization summarized scores to 0.0 \pm 0.0, while teleocidin B had an almost equal disruptive influence, yielding an averaged score of 0.4 \pm 0.6 (0.6 due to averaging of 2 scorers' measurements). Concentrations of TPA and teleocidin B at 0.5 ng/ml were also added to parallel cultures of villous adenoma 713. There was no morphological effect of this low dosage and no effect on actin organization. The scores were both 4.5 \pm 0.5, indistinguishable from the untreated control at 4.2 \pm 1.3. Another villous adenoma (Fig. 3a) also exhibited loss of actin organization when treated with teleocidin B (50 ng/ml) (Fig. 3g), yielding cultures similar in appearance to the primary adenocarcinoma shown in Fig. 3h. This mimicking of carcinoma actin organization is also shown by comparing the TPA-treated adenoma culture in Fig. 4b with the carcinoma cell line in Fig. 3j, and the teleocidin-treated adenoma culture in Fig. 4c with the adenocarcinoma in Fig. 3i.

DISCUSSION

In colon carcinoma development, cells lose actin cytoskeletal organization only when they become invasive. Benign colonic tumor cells exhibit a marked loss of growth control and can form

nodules several cm in diameter. Adenoma cells no longer respond to the growth controls operational in the normal colon which maintain crypt height at roughly 50 cells. The crypts in the adenoma can be hundreds of cells long, folding back over one another and sometimes branching to form a convoluted mass of tubules. Cells of the more advanced type of benign tumor, the villous adenoma, have also lost normal proliferative response to epidermal growth factor (urogastrone) (9). Nevertheless, adenoma cells in each of 12 benign tumors, covering a wide variety of histopathology classes, retained both an extensive intercellular and intracellular actin network. This tight network was only lost when the adenoma cells became malignant and capable of invading the muscle and fat layers surrounding the colonic epithelial layer.

We tested colonic tumors which had invaded the gut wall superficially (Dukes' A), moderately (Dukes' B), or extensively, reaching regional lymph nodes and sometimes extracolonic sites (Dukes' C). There was no statistically significant difference in the actin scores of these 3 classes. It must be emphasized that, although the Dukes' C class is diagnosed as metastatic, only the cells from the primary tumor in the colon, not those in the metastases, were assayed. Therefore, these results are not directly comparable to recent studies from 2 groups comparing low metastatic and high metastatic established cell lines by several parameters (18, 30). Low metastatic melanoma variant lines exhibited tightly packed intracellular actin bundles and restricted motility, while the high metastatic variants had few distinct actin bundles and high motile activity (30). These classes appear to correspond, respectively, to our 2-point and 0-point organization classes. All of the actin scores of our adenocarcinoma primary cultures fell between these values.

Loss of cytoskeletal actin organization may allow the colonic carcinoma cells to be more deformable than the rather rigid adenoma cells. Thus, the carcinoma cells and not the adenoma cells would be able to penetrate the colonic muscle and fat layers. Cells capable of rapid migration in culture have been shown to have fine actin microfilaments, while stationary cells exhibit actin stress fibers (13) of the type we have seen in adenomas. We assayed actin organization only after the epithelial cells had migrated from the explant to form a monolayer, not while they were forming the monolayer. After the partly digested crypts adhered to the culture dish, cells began to epibolize within a few hr and, after overnight culture, they had formed a continuous monolayer around the explant of several hundred to a few thousand cells. When the position of these epithelial patches

was marked, in a few cases to allow rapid inspection, it was not observed to change. We conclude that the epithelial patch cultures studied here are not noticeably motile. Our culture conditions thus favored the generation of actin stress fibers if the cells were capable of forming them. The adenoma cells formed stress fibers, while the carcinoma cells had, in most part, lost this ability.

The *in vivo* tissue organization is apparently maintained by our relatively gentle culture procedures. The tissue is not broken up into single cells, but cells are allowed to migrate from an explant as a continuous sheet. The colonic cells' ready migration onto the surface of the Petri dish is expected as they travel up the colonic crypt as part of their normal differentiation process. These migratory properties remain even when the differentiation process which normally accompanies them is aberrant. Epithelial cells cultured from adenomas remain in a tissue-like network, as they remain attached by junctional complexes and extensive membrane interdigitations along their lateral surfaces (9). Large groups of neighboring adenoma cells maintain an intercommunicating network *in vitro*. About 50 adjacent epithelial cells were able to transmit fluorescein dye molecules to one another, presumably through gap junctions (10).

The majority of cells assayed for actin organization were originally within the tumor and did not result from cell division *in vitro*. Most benign tumors (11 of 12) were cultured for no more than 4 days, a little over one complete cycle, which is estimated to be 2.5 days *in vitro* (9). The majority of carcinoma cultures (6 of 7) were cultured for no more than 3 days. Only a subpopulation of cells in either adenoma or carcinoma primary cultures divided *in vitro*, as only one-fourth to one-half of the cells in such cultures incorporated [³H]thymidine in continuous labeling experiments (9).⁴ Therefore, a large proportion of both preneoplastic and malignant cells which were assayed for actin organization in culture had migrated from the tumor without DNA synthesis. Cells allowed to epibolize from the explant for as short a period as 18 hr retained the same actin organization as did those cultured for longer period. Carcinomas 574, 711, and 717 and adenomas 572 and 558 were fixed after only an overnight culture (listed as 1 day in Table 1). In each case, the carcinomas displayed a loss of most actin cables, while the adenomas retained significant cytoskeletal organization. Similar results were seen after 5 to 6 days of primary culture. However, no actin cables were seen in any colon carcinoma cell line studied, suggesting that the rigors of continuous culture select for cells with further disorganized actin cytoskeletons.

In the murine fibroblast-viral transformation model of carcinogenesis, PA secretion has been linked to tumorigenicity, anchorage-independent cell growth, and disruption of the actin cytoskeleton (24). What role is played *in vitro* and, possibly, *in vivo* by the release of PA from late-stage premalignant cells within adenomas? The major effect of PA is a loss of the tight tissue-like organization which characterizes adenomatous epithelial cells. We can cause the release of PA by addition of promoting agents such as TPA or teleocidin to such epithelial cells in primary culture. TPA also induces a PA in normal chick embryo fibroblasts. In similar fashion to the data presented here, the chick PA induces loss of ordered actin-containing structures, mimicking the transformed phenotype (25). In the human cell systems, both the actin cytoskeletal network (this study) and the gap junction network are disrupted (10). Gap junctions interconnect

adjacent cells, allowing extensive intercellular communication in the tissue.

TPA-treated premalignant cells are phenotypically identical by these 2 criteria to malignant cells in primary culture. However, this similarity may be misleading. There is no evidence that TPA induces the activation of an oncogene which would catalyze the benign-to-malignant transition. It may be that the secretion of plasminogen-activator *in vivo* functions primarily to cause focal disruption of the tightly organized epithelial tissue of the benign tumor around a newly emergent malignant cell. We hypothesize that the induction is caused by an endogeneous tumor promoter. A polypeptide released by transformed cells has been shown recently to induce PA from fibroblasts (3). A similar factor in the colon might act as an endogeneous promoting agent. Activation of a cellular oncogene in a late-stage premalignant cell would convert it to malignancy. However, by this hypothesis, its malignancy would remain suppressed until it was freed of the growth control exerted by the surrounding benign tumor cells. The situation may be analogous to the fate of the teratocarcinoma cells injected into a mouse blastocyst. The malignant cell provides genetic information to the development of the normal mouse because its malignant properties are suppressed by the surrounding normal cells in the early mouse embryo (19, 22). The carcinoma cells arising within an adenoma may be constrained similarly and unable to proliferate independently of the surrounding premalignant cells. The malignant cell, by this model, would become free by literally losing contact with the surrounding adenomatous cells by local membrane digestion with plasminogen activator. This digestion would disrupt both intercellular communication by destroying gap junctions and interrupting the intercellular actin network. The collapse of intracellular cables within the malignant cell would occur, leading to the reorganization of actin monomers into a network under the plasma membrane. In fact, most actin fluorescence is seen as a diffuse band under the membrane in transformed cell lines (Fig. 3j). The freeing of a malignant cell would not necessarily be a 1-step event. Several exposures to PA may be necessary to generate a focus of carcinoma cells large enough to replicate autonomously.

This hypothesis is not without some experimental support. The presence of infiltrating carcinoma within an adenoma had no measurable effect on actin organization. Infiltrating carcinoma, not merely a focus of carcinoma, was found within both villous adenomas 558 and 708, yet the actin organization values of these cultured benign tumors were, respectively, 3.9 ± 1.1 and 4.7 ± 0.7 (Table 1). These are statistically indistinguishable from the values seen in the least advanced preneoplastic cultures—the 2 tubular adenomas without any dysplastic cells (immediate precursors of carcinoma), 616 and 690. These adenomas scored 4.2 ± 1.5 and 4.0 ± 1.5 , respectively. Perhaps the strong structural support of the actin network interconnecting the adenomatous preneoplastic cells has to be broken for the malignant cells among them to express their characteristic cytoskeletal abnormalities, leave the adenoma, and invade the colonic muscle layers. We will attempt to resolve this issue by first identifying the cultured carcinoma cells within the adenoma-derived epithelial patches from tumors such as 558 and 708 using our panel of monoclonal antibodies. These antibodies bind to the cell surface of the majority of carcinoma cells in primary culture, but to only a few, if any, adenoma cells (data not shown). Because we have rhodamine-conjugated phalloidin and fluorescein-labeled monoclonal antibodies, we can study the same cell using

⁴ E. Friedman, unpublished data.

both probes.

The maintenance of the actin cable pattern through these genetically different preneoplastic adenomatous stages until transition to carcinoma suggests that the cytoskeleton is the site of action for an oncogene product which acts at a late stage in tumor development. Cell-shape changes characterize the transformation of NIH3T3 cells by certain cellular oncogene products (27). We hypothesize that the adenoma-to-carcinoma transition is mediated by a similar gene product. Highly purified pp60 src protein has been shown to disrupt actin stress fibers when microinjected into normal fibroblasts (17). The location of pp60 src and p21 ras proteins under the cell membrane (2, 16, 31, 32), in close proximity to the actin cytoskeleton, makes these 2 oncogene products possible candidates for genes activated only at late premalignant stages, such as the villous adenoma-to-carcinoma transition in the colon.

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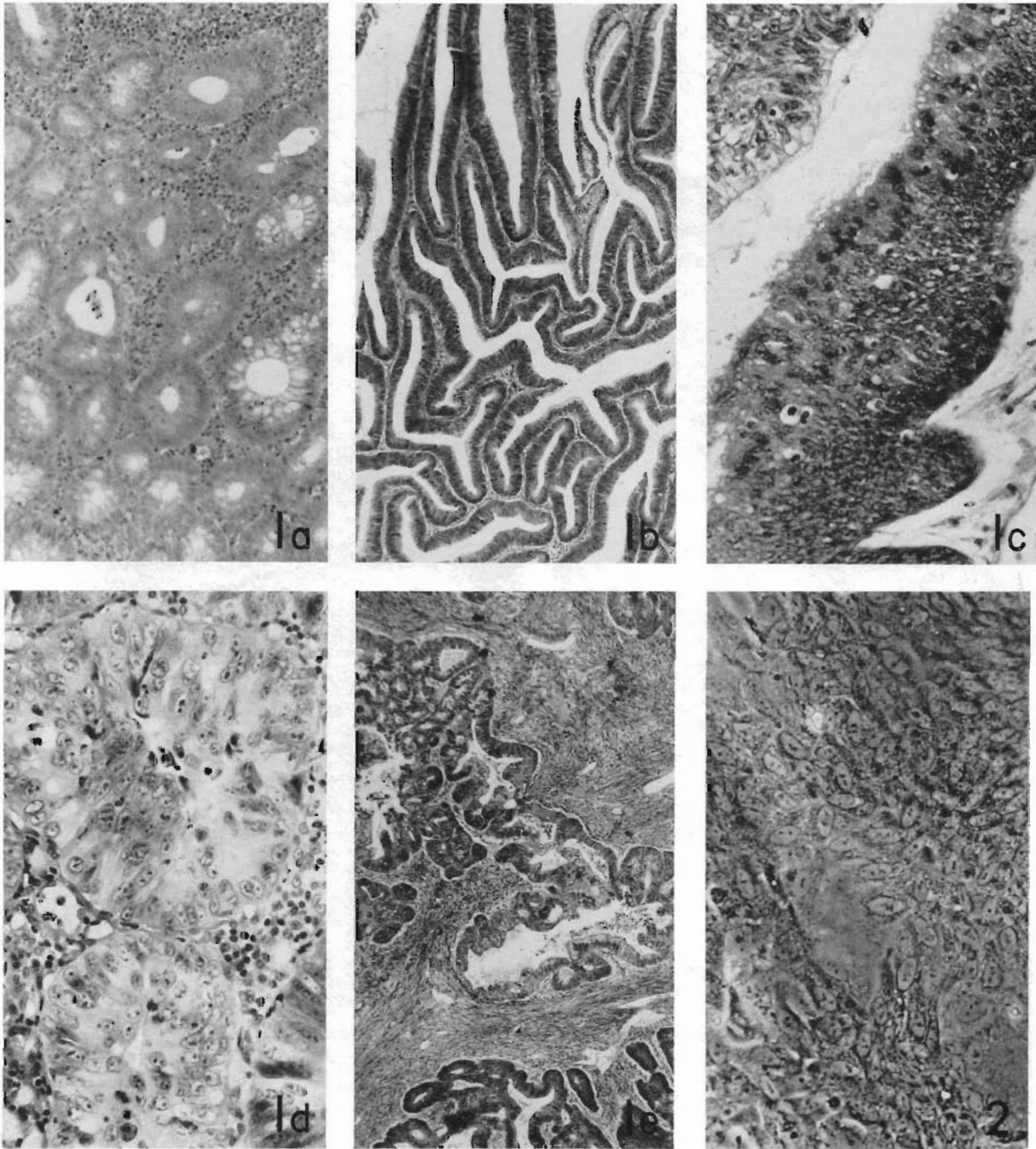


Fig. 1. Representative sections of fixed human preneoplastic and neoplastic tissues. a, cross-section of tubular adenoma showing rounded crypts. $\times 40$. b, cross-section of villous adenoma showing serrated crypts. $\times 40$. c, focus of severe dysplasia within an adenoma showing multilayering of cells. $\times 100$. d, an area of carcinoma *in situ* within an adenoma showing typical back-to-back abnormal crypts with no intervening lymphocytes. $\times 100$. e, adenocarcinoma infiltrating into the muscular layer surrounding the epithelial layer of the colon. $\times 40$. All sections were embedded routinely and stained with hematoxylin and eosin.

Fig. 2. Colonic epithelial patch. Phase-contrast photomicrograph of a monolayer of living colonic epithelial cells. These cells have migrated as a tightly packed sheet from an explant of adenoma tissue. $\times 97$.

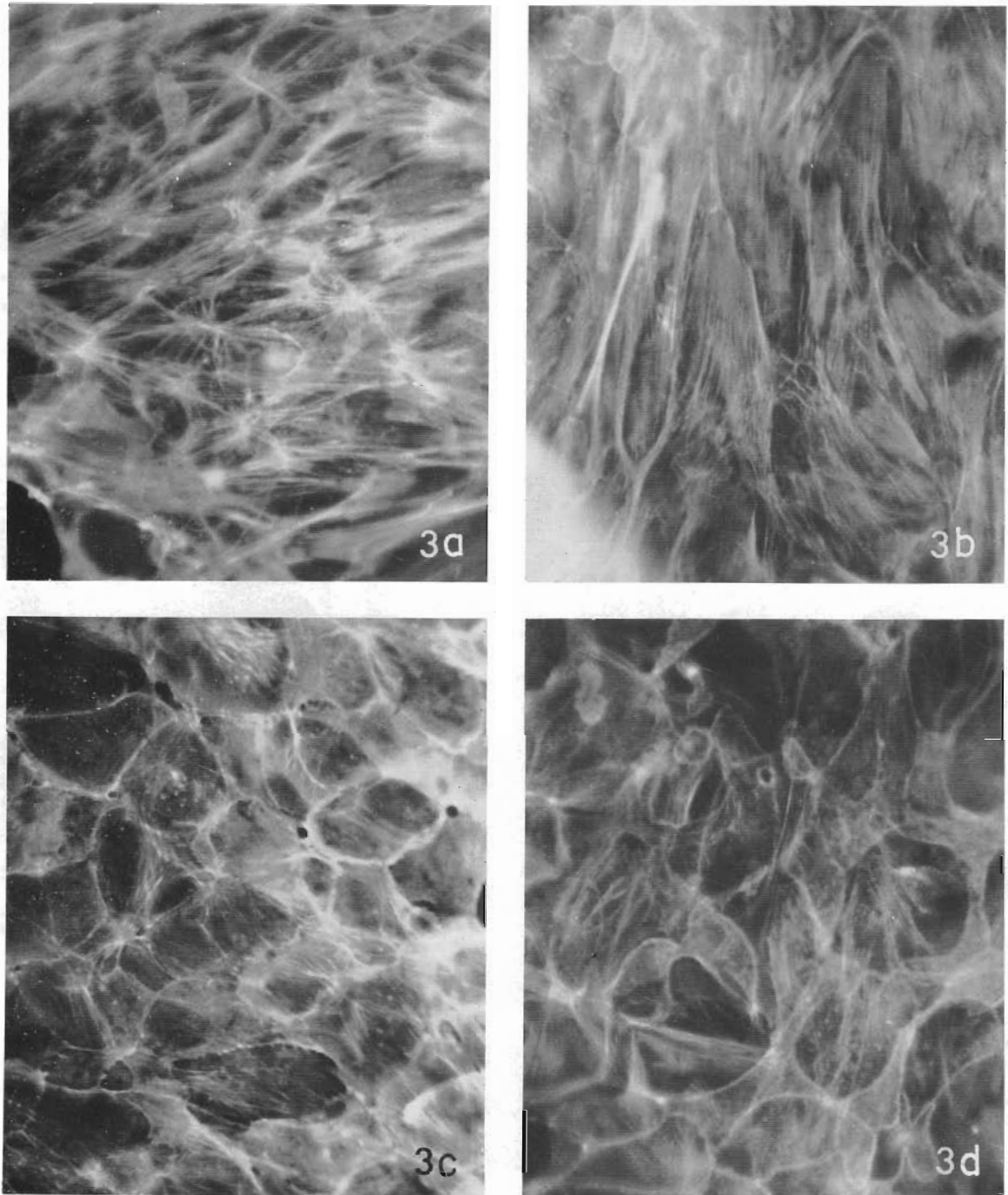
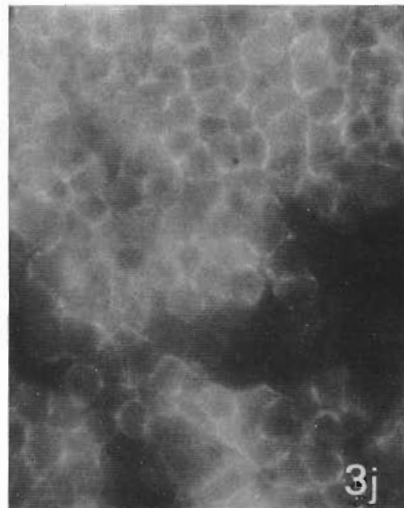
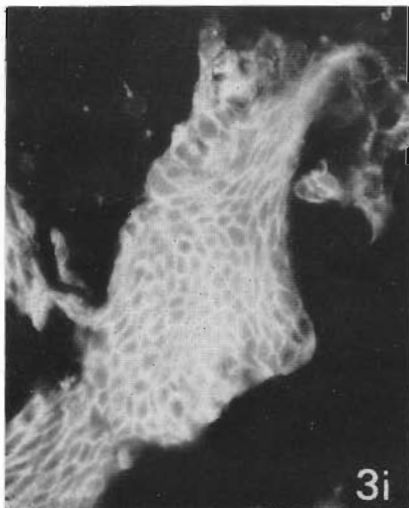
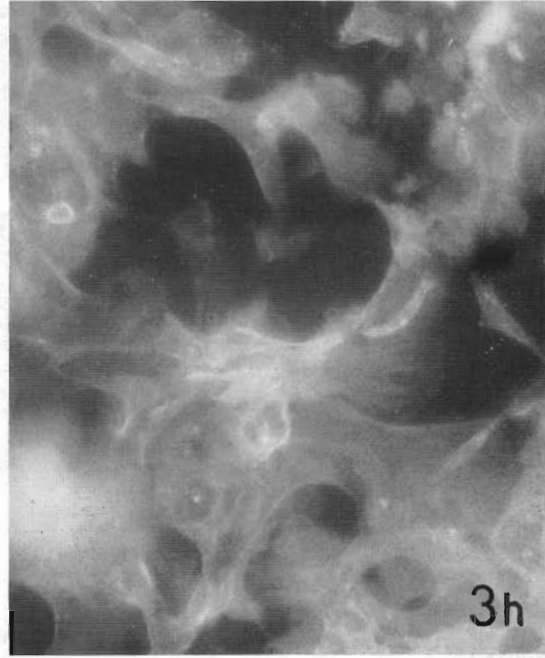
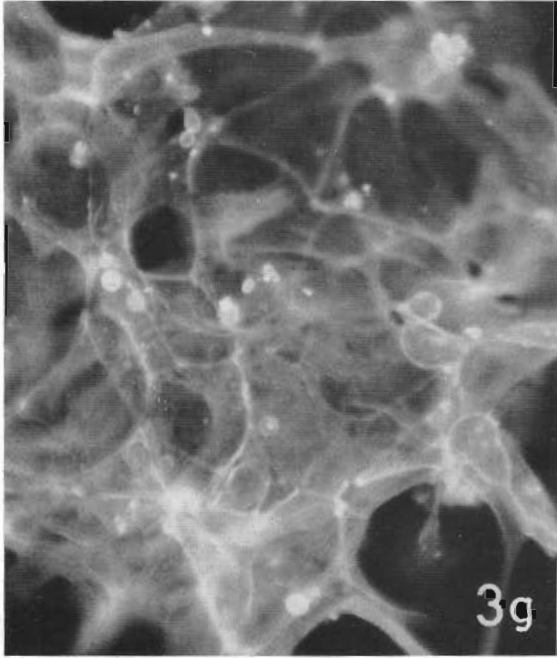
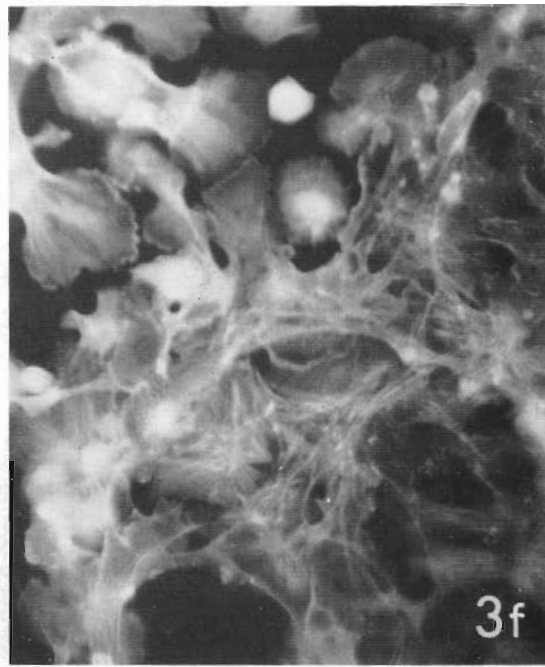
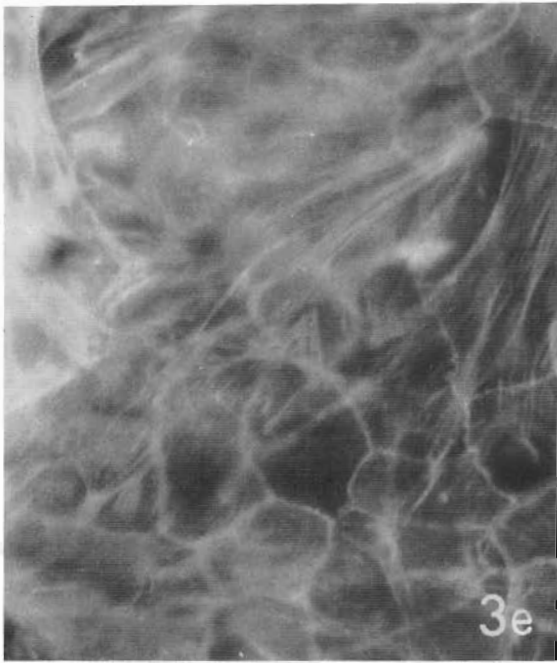


Fig. 3. Categories of actin cytoskeletal organization. Adenomas and carcinomas were placed in primary culture as described (except for the cell line in *j*), permeabilized, and stained with rhodamine-conjugated phalloidin, and representative areas were photographed in the culture dish ($\times 1630$; *i* and *j*, $\times 815$) ("Materials and Methods" and Table 1). For description of categories, see "Materials and Methods." *a*, late-stage preneoplastic (PA-secreting) villous adenoma 708 showing intercellular actin network connecting a group of adjacent cells in primary culture. Intracellular cables are also seen crossing the cells (5 points). *b*, early-stage preneoplastic (not secreting PA) villotubular adenoma 668 showing extensive intercellular network almost obscuring individual cells. Intracellular actin cables are also present (5 points). *c* and *d*, less actin organization seen, as the intercellular network is largely absent, but cells retain many intracellular cables, and much actin delineates the cell peripheries; *c*, villous adenoma 558; *d*, villotubular adenoma 674 (both 4 points). *e*, less cytoskeletal organization is exhibited by villotubular adenoma 588 (3 points). *f*, PA secretion induced by TPA from villous adenoma 676 is inhibited to some extent by 1 mM benzamidine present in the medium (2 points). Compare with Fig. 4*b*, in which no benzamidine was present and the actin score was reduced to 0. *g*, villous adenoma 708 (untreated parallel culture shown in *a*) treated for 24 hr with teleocidin B (50 ng/ml) (1 point). *h*, adenocarcinoma 574 in primary culture, 1 day (1 point). *i*, adenocarcinoma 711 in primary culture 1 day, (0 points). *j*, human colon carcinoma cell line SW1116 (0 points).



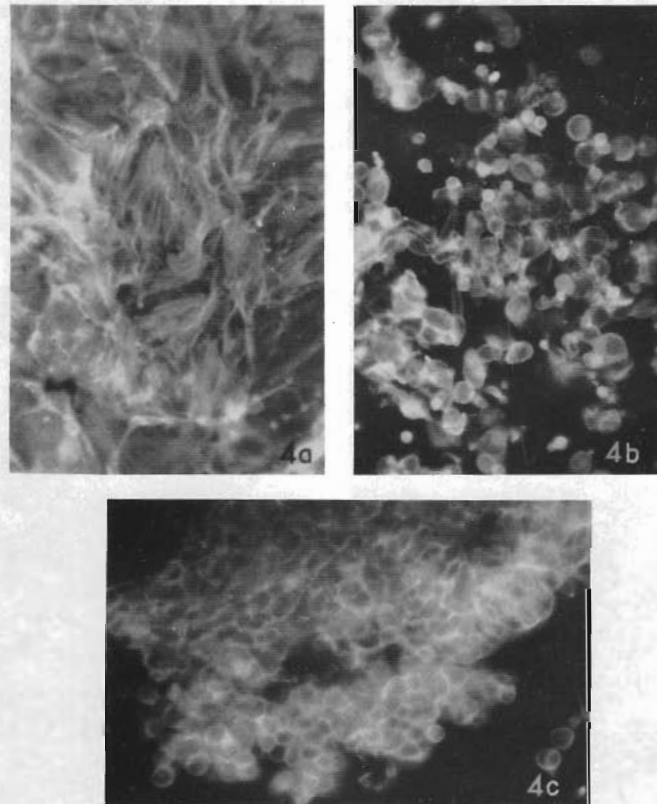


Fig. 4. Loss of cytoskeletal actin organization by induction of PA in a cultured villous adenoma by the promoting agents TPA and teleocidin B. After 3 days of growth, parallel cultures of adenoma 713 were treated for 24 hr with TPA (50 ng/ml), teleocidin B (50 ng/ml), or 10^{-7} M deoxycholic acid or were left untreated. The cultures were fixed, permeabilized, and stained with rhodamine-conjugated phalloidin, and representative areas of each culture were then photographed ("Materials and Methods"). The deoxycholic acid-treated culture was identical to the control and so not shown ($\times 815$). a, villous adenoma 713, untreated control (5 points). b, villous adenoma 713 parallel culture exposed to TPA (50 ng/ml) 24 hr, showing loss of cytoskeletal organization (0 point). c, villous adenoma 713 parallel culture exposed to teleocidin B (50 ng/ml) 24 hr, showing loss of cytoskeletal organization (0 point).