Altered Actin Cytoskeletal Patterns in Two Premalignant Stages in Human Colon Carcinoma Development

Eileen Friedman,1 Michael Verderame, Martin Lipkin, and Robert Pollack2

ABSTRACT

Primary culture of human colonic biopsies converts the single cell thcck epithelial layer from a highly indented sheet in vivo into a flat patch on the surface of a Petri dish. Migration of cells from biopsies in a continuous sheet to form the patch cultures allows the cultured cells in large part to retain the junctional complexes and membrane interdigitations which connect adjacent cells in vivo and therefore to maintain their spatial relationships to neighboring cells. Migration of the cells onto a flat surface also allows visualization of their actin cables (E. Friedman, M. Verderame, S. Winawer, and R. Pollack, Cancer Res., 44: 3040-3050, 1984). Actin organization patterns have been studied in primary patch cultures of colonic epithelial cells from four stages in the development of colon cancer: normal tissue, normal-appearing but preneoplastic cells characteristic of familial polyposis patients, benign tumors or adenomas from familial polyposis patients, and benign and malignant tumors from patients in the general population. Carcinomas exhibited the least number of actin cables, while adenomas contained the greatest concentration. Similar actin patterns were seen in both familial polyposis and nonpolyposis adenomas. The preneoplastic prebenign tumor stage characteristic of familial polyposis patients had less actin cables than either normal cells or benign tumor cells. Thus actin organization loss characterized the transition from the normal colonic epithelial cell to the preneoplastic nontumor cell. The ability to form actin cables was then regained with the transition from the preneoplastic prebenign tumor cell to the benign tumor cell and lost again with the benign tumor to malignant tumor transition. The complexity of these changes in actin organization during the step-wise transformation of colonic epithelial cells was not predicted from the simple model of actin cable loss accompanying fibroblast transformation.

INTRODUCTION

Comparison of normal and neoplastic cells has revealed several biological differences measurable in vitro. In fibroblast systems, a common marker for transformation has been a loss of actin cables. In an earlier study (1), we found that actin cables were unexpectedly found in one kind of human colonic tumor, the benign tumor or adenoma. It is the direct precursor of the malignant tumor or carcinoma during the development of this solid tumor (2, 3). Actin cables were largely absent from colonic carcinomas in primary culture, in contrast. The paradigm of actin cable loss as a marker of colonic epithelial cell transformation was then modified to state that it is a marker of the transition between a noninvasive tumor, the adenoma, to the invasive carcinoma.

Recently, methods have been developed to reproducibly place into primary culture both normal colonic epithelial cells from adults and preneoplastic cells from nontumor areas of the colons of familial polyposis patients (4). It was then possible to study in culture the evolution of colon carcinomas through these two earlier stages. If the fibroblast model was predictive of the events in epithelial cell transformation, both normal and nontumor preneoplastic familial polyposis cells would contain numerous actin cables, as did the benign tumor. The loss of actin cables would then be a very late event in transformation. The picture that emerged in the current study was more complex and indicated that the actin cytoskeleton was modified at several stages in tumor development.

MATERIALS AND METHODS

Tissue Culture of Colon Epithelial Cells and Actin Cytoskeletal Visualization. Cells were placed into identical conditions of primary culture for one day. They formed epithelial patches by migration from small explants onto gelatin-coated 35-mm dishes (Coming) as described (4-6); they then were fixed with formalin, permeabilized, and stained with rhodamine-conjugated phalloidin as described (1).

Quantitation of Actin Organization. Random fields of each epithelial patch were photographed at an objective magnification of 40×, and two photographs (4 × 5 inches) were printed from each frame. They were number-coded on the back and scored independently by two observers without knowledge of the source of the tissue being scored. Each photograph was placed into one of 6 categories based on the morphology of the monolayer and the presence, size, and distribution of actin cables, as described more fully in the earlier reference (1), using a scale from 0 (no cables) to 5 (extensive heavy cable network). The mean and standard deviation of the scores for all the photographs of each specimen were then compiled.

RESULTS

Stages in Colon Carcinoma Development Assayed. Colonic epithelial cells at different stages in carcinoma development have been used in this study. Several were biopsied from FPS3 patients or their FPA relatives. Familial polyposis is a disorder inherited as an autosomal dominant which very strongly predisposes the patient to develop colon cancer and to develop it at an early age compared with the general population (7). The name derives from the appearance of the patients' colons, which were studded with hundreds to thousands of polyps, hence polyposis. These polyps are benign tumors or adenomas. They are the direct precursors of carcinomas which begin as foci within them.

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The abbreviations used are: FPS, symptomatic with familial polyposis; FPA, asymptomatic but at risk to develop familial polyposis; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.
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Because of the large number of these lesions and their early appearance while the patient is in his teens to 30s, it is inevitable that one of the adenomas develops into a carcinoma during the patient's lifetime. The adenoma is not the earliest lesion, however. Before adenomas develop, other changes have been found to characterize areas in the colonic epithelium. The colonic epithelium is a monolayer in vivo which is correted into test tube-like indentions into the gut wall, called colonic crypts. The proliferating cells in normal subjects are limited to the lower two-thirds, while the cells at the top of the crypt are terminally differentiated. In the colons of familial polyposis patients, this pattern of differentiation is both altered and delayed (8). Dividing cells are found all along the crypt length. The crypt is shortened as the abnormally differentiated cells at the crypt top and gut lumen tend to exfoliate more rapidly than normal. The presence of abnormally fragile cells at the top of the crypt may explain the ready fragmentation of a mucosal biopsy from a familial polyposis patient into individual crypts (Ref. 4; also shown in Fig. 1c giving rise to a monolayer by cell migration). They are histologically indistinguishable from familial polyposis adenomas. Thereby appearing to unite neighboring cells into a multicellular network (Fig. 1, g to i). The nonpolyposis adenoma (Fig. 1j) was indistinguishable from the other sporadic adenomas studied earlier (1). To ensure that our methodology was consistent from the earlier work, a carcinoma in primary culture from a nonpolyposis patient was also examined and was found to resemble carcinomas studied earlier (Fig. 1j). It exhibited a loss of actin cables (average carcinoma score, 0.9; Table 2). Its actin-containing structures appeared as a halo of tiny filaments at the inner surface of the cell membrane in contrast to the adenoma pattern.

Benign Tumors from Polyposis and Nonpolyposis Patients. Colonic epithelial cells from three benign tumors from FPS patients and one nonpolypsis patient were placed into primary culture for a period of one day and then permeabilized and stained for the presence of actin cables ("Materials and Methods"). Actin cytoskeletal organization patterns in adenomas from both types of patients were indistinguishable (P = 0.18; Table 2), with mean actin scores of 4.70 and 4.33, respectively (Table 2). Cells from both sources had numerous thick intracellular actin cables which ran into foci at the cell peripheries of adjacent cells, thereby appearing to unite neighboring cells into a multicellular network (Fig. 1, g to i). The nonpolyposis adenoma (Fig. 1j) was indistinguishable from the other sporadic adenomas studied earlier (1). To ensure that our methodology was consistent from the earlier work, a carcinoma in primary culture from a nonpolyposis patient was also examined and was found to resemble carcinomas studied earlier (Fig. 1j). It exhibited a loss of actin cables (average carcinoma score, 0.9; Table 2). Its actin-containing structures appeared as a halo of tiny filaments at the inner surface of the cell membrane in contrast to the adenoma pattern.

Normal Cells and Premalignant Epithelial Cells from Non-tumor Tissue. We placed into primary culture for 1 day normal colonic epithelial cells from 5 control subjects genetically at low risk to develop colon cancer because of a generation-free family history of colon cancer (4), nonpolyposis early-stage preneoplastic cells from 9 FPS patients, and histologically normal cells from 4 patients in familial polyposis families at risk to develop the disease but as yet asymptomatic (FPA). These normal and early-stage premalignant cells, like adenoma cells, are fairly thick, as they are cuboidal to columnar in vivo and in vitro (4). We focused at various depths in each cultured monolayer but never saw the thick cables characteristic of adenomas in any of these cell types. Fine, thin cables were seen in certain normal and FPA cells (Fig. 1, a to d) at some depths of field, but they never stretched completely across cells as they did in the adenoma cells (Fig. 1, h and i). The cytoplasm of each normal or FPA cell appeared full of fluorescent material which was not resolvable into filaments at a constant plane of focus. This intracellular fluorescence was not due to poor washing as the background Petri dish was black (see Fig. 1a, upper corner) and there was no fluorescent halo around the cells. The fluorescence was more distinct at the borders of the cells, outlining them. The cytoskeletons in these cells looked like the partially unravelled adenoma actin cytoskeletons observed in our earlier studies. Adenoma cells which had been treated with both TPA to induce the protease, plasminogen-activator, which depolymerized actin ca-
bly expressed and had a partially disrupted actin cytoskeleton (see Ref. 1, Fig. 3f). In the absence of the inhibitor, the adenoma actin cables completely disappeared (Ref. 1, Fig. 4b). The partially polymerized actin cables in the protease-inhibitor-treated case gave the adenoma cells a non-resolvable fluorescence all through the cytoplasm, as was seen in the current study with normal cells. In the earlier report, this state had been graded with an actin score of 2 on a scale of 5 for heavy, cell-spanning cables and 0 for no cables. A majority of areas within untreated adenomas also had few cables and had been scored as grade 3 (see Ref. 1, Fig. 3e). Most normal cells and FPA cells fell between these categories, with mean actin organization scores of 2.66 ± 0.42 and 2.00 ± 0.29, respectively (Table 2). There was no statistical difference between these values (P = 0.27). Therefore, actin organization patterns did not differ between normal subjects and those who may have inherited the FPS gene(s) but who have not yet shown any clinical signs of developing familial polyposis.

FPS nontumor preneoplastic cells, in contrast, had less intracellular fluorescence than did either normal or FPA cells, and few, if any, thin actin filaments discernible in the cytoplasm. Filaments were seen predominately at the periphery outlining the cell membrane (Fig. 1, e and f). The mean actin organization score of the FPS monolayers was 1.67 ± 0.2, a statistically significant decrease compared to normal cells (P = 0.016; Table 2). There was an overlap in some values for these two groups, so actin organization as scored in this study does not unambiguously distinguish an FPS patient from a normal subject. The test in itself yields a reproducible measurement of each patient’s cells. Two familial polyposis biopsies, 897 and 917, had been taken from one patient at a 2-month interval and gave very similar scores of 1.6 and 1.5 (Table 1). The repeated biopsy was not known to the scorers until the code was broken. The generally weaker actin cytoskeleton in FPS nontumor cells may explain why the colonic epithelial layer in these patients tends to fragment readily into crypts, in contrast to the epithelial layer in normal patients (Fig. 1k; Ref. 4). A single layer of epithelial cells at the opening of the crypts into the gut lumen holds adjacent crypts together. Fragile cells due to a weaker-than-normal actin cytoskeleton, if present at the top of FPS colonic crypts, would easily fragment when the tissue was handled.

FPS preneoplastic cells are believed to be the direct precursors of the simplest histological class of adenoma (4, 7). The difference in actin organization between the normal-appearing preneoplastic FPS cells and adenoma cells was very clear, both by visual inspection and by scoring. The scores were, respectively, 1.6 ± 0.2 (SD) and 4.7 ± 0.1, a difference very statistically significant with a calculated P value of 7 x 10^-7 (Table 2). This difference was further documented by controlling interindividual variation and studying both adenoma cells and preneoplastic nontumor cells from the same FPS patient, Patient 917. His adenoma (Fig. 1, g and h) gave a characteristically high actin score (4.8), while his nontumor preneoplastic cells (Fig. 1, e and f) scored quite low (1.6). Therefore, a second change in actin cytoskeletal organization pattern occurred between premalignant stages in colon carcinoma evolution.

**DISCUSSION**

Four different patterns of actin cytoskeletal organization were observed in colonic epithelial cells at different stages of human colon cancer development. An extensive actin cable network characterized only the benign tumor stage, while few actin cables were seen in normal cells, pretumor preneoplastic cells, or carcinoma cells under identical conditions of short-term primary culture. The tissues these epithelial cells were derived from were clearly distinguishable by their histopathology and, in the case of FPS patients, also by their clinical diagnosis and family history. The cultured epithelial cells derived from these tissues had been shown to differ in various properties in earlier studies. The phorbol ester tumor promoter TPA induced mitogenesis of FPS nontumor cells and the simplest class of adenoma cells but not normal cells. In contrast, TPA induced the secretion of the protease plasminogen activator from each carcinoma in primary culture and the more advanced adenomas (4, 6). Therefore, the pathology of the tissues placed into culture, the properties of the cultured cells, and the actin cytoskeletal organization patterns all served to distinguish the 4 cell types under study.

The human keratinocyte and its transformed derivatives and human colonic epithelial cell stages in carcinoma development show similar actin patterns, with few actin cables in normal and carcinoma cells but many thick cables in a precarcinoma tumor state. Sectioned normal human epidermal cells and the corresponding cutaneous carcinoma displayed very similar actin patterns, with the actin filaments predominately at the cell periphery (9). Human keratinocytes did not display actin cables, even when cultured. Cables were only seen in the postcrosis period after simian virus 40 transformation (10). The simian virus 40 keratinocyte transformants were immortal and grew in soft agar (10) but did not form progressively growing tumors in nude mice. Possibly these noninvasive viral transformants are the equivalent in this keratinocyte transformation system of human benign colonic tumors.

These modulations of the actin cytoskeleton at different stages of human epithelial cell transformation in both skin and colon were not seen when murine mammary cells were examined. Normal tissue, hyperplastic alveolar nodules, and primary breast neoplasms were digested to single cells and small clumps, plated on glass coverslips, and then allowed to divide until 50 to 60% confluent at 2 to 4 days postseeding. The cultured cells were then permeabilized and stained with anti-actin antibodies and examined by indirect immunofluorescence. Most of the cells in each primary culture contained actin cables (11). The differences between these studies may lie in the cell types examined or the species, mouse versus human. Alternatively, it may reflect the equally good adherence of murine mammary epithelial cells from the 3 tissue types on glass coverslips.

The possibility exists that the normal and FP preneoplastic pretumor cells could form actin cables if they adhered as tightly to the gelatin-coated culture dish as adenomas and carcinomas. Tight attachment to the substratum is obviously not a sufficient condition as the tightly attached carcinoma cells still did not form actin cables. Looser adherence by normal and pretumor cells might be a reflection of their in vivo property of shedding terminally differentiated cells at the gut lumen. This occurs at such a high rate that the entire epithelial layer of the colon is renewed in about a week. The adenomas and carcinomas, in contrast, form very elongated crypts because the rate of cell loss, although considerable, is not equal to the rate of cell renewal, and the
controls of crypt length at about 50 cells in the normal state are lost in tumors. An alternative explanation for the difference in cell adherence to the Petri dish may lie in the need by normal cells for a defined substratum for attachment, while the tumor cells may have a decreased need for a basement membrane and can make do with gelatin. On the other hand, the decreased adherence may be caused by the FPS and normal cells’ inability to make actin cables. The weakened actin cytoskeleton in FPS pretumor cells compared to normal cells may explain why the FPS colonic mucosa tends to fragment into single crypts (Fig. 1k) and the crypts are shortened. The abnormally differentiated cells at the top of the crypt may be so fragile that they shed more rapidly than normal, producing shorter crypts. When the tissue is manipulated, these cells also tend to fragment, releasing individual crypts.

Fibroblast transformation systems are not characterized by stable intermediate stages that can be convincingly correlated to premalignant stages in vivo. This data is not available for human sarcoma formation. However, there are reports in the literature that changes in actin organization patterns or the actin molecule itself in fibroblasts may indicate a predisposition to transformation. Familial polyposis is a systemic disease in many affected individuals, with abnormalities observed in skin, bone, teeth, stomach, and duodenum (12), as well as the colonic epithelium. Dermal fibroblasts from FPS individuals contained fewer organized microfilaments compared to skin cells from normal individuals (13, 14). A mutation in a β-actin gene in a normal human fibroblast strain was induced by a chemical carcinogen (15, 16). Morphologically transformed cells were not observed, however, until after extensive subcultivation and at least 13 cell generations, by the author’s calculation (17). The quantity of the mutated β-actin was also found to be reduced in human sarcoma formation. However, there are reports in the literature, such as actin-associated molecules or the actin cytoskeleton in FPS colonic mucosa, that may not be actin molecules themselves. The modification in the actin cytoskeleton in FPS colonic cells may be caused by any of a number of sources, such as actin-associated molecules or other regulatory molecules. The parallel between this study and those of Leavitt and Kakunaga (15–18), we believe, is that modifications in the actin cytoskeleton occur at least 2 points prior to colon carcinoma development. In the first stage studied here from FPS patients, the cells are morphologically grossly normal, without a tumor phenotype, similar to the human dermal fibroblasts shortly after carcinogen treatment. The tumor phenotype occurs only when the FPS cells undergo further, presumably genetic, changes. These occur in the body over a long period of time, months to years. Similarly, several cell generations were needed to develop a tumor phenotype from carcinogen-treated human dermal fibroblasts, suggesting the need for those cells to pass through premalignant stages before the malignant state was reached.

REFERENCES

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Fig. 1. Colonic epithelial cells at 4 stages in the evolution of colon carcinoma were placed into primary culture for 1 day and then stained for the visualization of actin cables ("Materials and Methods"). All photomicrographs except k are at a 297× final magnification. a and b, normal cells from Patient 900 genetically at low risk to develop colon cancer; c and d, FPA Patient 922, phenotypically normal cells; e and f, pretumor neoplastic cells from FPS Patient 917; g and h, premalignant benign tumor cells from FPS Patient 917; i, premalignant benign tumor cells from nonpolyposis Patient 857. The field was selected to show the high frequency of actin cables which obscure individual cell outlines; j, carcinoma 861; k, single colonic crypt from an FPS patient giving rise by migration to a sheet of epithelial cells; final magnification, ×30.
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