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Fodrin as a Differentiation Marker

Redistributions in Colonic Neoplasia

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Fodrin (nonerythroid spectrin) is a 475,000 molecular weight (MW) (apparent) heterodimeric actin-binding protein usually found in mature cells at the cytoplasmic face of the plasma membrane. While its precise role is uncertain, it may participate in the establishment and/or maintenance of cell polarity, shape, and specialized receptor domains. In polarized epithelial cells, an asymmetric distribution of fodrin appears to signal phenotypic maturity. Using immunohistochemical techniques, the distribution of fodrin in enterocytes during normal crypt-to-villus maturation, and in adenomas, adenocarcinomas, and cultured Madin–Darby Canine Kidney (MDCK) cells has been studied and its abundance quantitated by immunoblotting and digital immunofluorescent confocal microscopy. During normal maturation, fodrin was found to assemble at the apex of the enterocyte, presumably in the terminal web, only in those cells near the villus tip. Villin was found in an apical location in both crypt and surface enterocytes. In adenocarcinomas of the colon (n = 11), there were enhanced levels of fodrin at the apex, and an approximately threefold increase in the total amount of fodrin per cell relative to normal crypt enterocytes. An increased percentage of this protein was also found in the cytoplasm. Adenomas (n = 7), nonconfluent MDCK cells in culture, and two (of two) cases of ductal carcinoma of the breast also demonstrated enhanced cytoplasmic and total fodrin. Supranormal levels of fodrin at the apex of enterocytes were also observed in Crohn's disease samples and in the normal-appearing enterocytes adjacent to a tumor. It is hypothesized that increased apical fodrin may signal a reaction of the microvillar brush border to pathologic stress, while increased cytoplasmic and total pools of fodrin may mark neoplastic activity. These findings may be of diagnostic value, particularly in the evaluation of small biopsies or cytologic material. (Am J Pathol 1989, 135:1197–1212)

The recognition of phenotypic change at the histologic level is the foundation of modern pathologic diagnosis. Yet, with the possible exception of studies on intermediate filament isoform switching,[1] scant attention has been given to the molecular changes specifically underlying even the most commonly observed morphologic alterations in diseased cells.[2] Indeed, most tumor or tissue markers, while aiding in the recognition of such cells, are unrelated to the actual proteins that affect the pathologic changes in cell morphology.

One important determinant of epithelial cell appearance is the fidelity of their polarization and the appearance of specialized structures on specific surfaces. Classically, polarized epithelial cells have at least four membrane domains: 1) an apical region, which in the enterocyte is composed of the microvillar brush border; 2) the region of the zonula adherans and the zonula occludens, which separates the apical from the basolateral domains; 3) the lateral domain; and 4) the basal domain, which is the region of the epithelial cell in closest contact with the substratum.[3,4] While the mechanisms that establish and maintain these domains are incompletely understood, it is clear that each contains characteristic proteins, and that a group of proteins that collectively form the cortical cytoskeleton are involved. Thus, in the human enterocyte, the apical brush border contains the cytoskeletal proteins villin, fimbrin, brush-border myosin I (also called 110K-CM), fodrin (nonerythroid spectrin), a cytoplasmic myosin, and tropomyosin.[5–7] The tight junction contains ZO1, and cingulin[8,9]; the lateral domain contains fodrin and ankyrin, linked to α-Na,K-ATPase[10–12]; and the basal domain

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contains talin, vinculin, and others linked to the substratum *via* the integrins.[4]

Previous studies have examined changes in the distribution of villin, a cytoskeletal protein confined to the apical domain of primarily intestinal and renal epithelial cells, during both normal enterocyte development[5,13–15] and in precancerous and cancerous colonic lesions.[5,6,16,17] These studies established the value of villin as a marker of intestinal or renal epithelium, but demonstrated that the assembly of villin at the apical membrane surface was preserved in all but the most anaplastic carcinomas.[6,16,17]

Another cytoskeletal protein associated with the cytoplasmic face of the membrane is fodrin (also known as nonerythroid spectrin[18,19]). Fodrin is an actin binding protein composed of two similar but nonidentical subunits of 240,000 and 235,000 apparent molecular weight (MW). The actual MW of the α subunit is 286,000, based on the derived amino acid sequence.[20] It is one of the best studied members of a class of proteins now termed the spectrin superfamily,[21] which includes the erythroid and nonerythroid spectrins, α-actinin, and dystropin. In the mammalian erythrocyte, spectrin contributes to the shape and stability of the cell; in nonerythroid cells, fodrin appears to play a central role in establishing and maintaining cell polarity,[10–12,19,22] in mediating exocytosis in chromaffin cells,[23] and in maintaining and possibly regulating organized receptor domains on the plasma membrane.[10–12,19,22,24] Significantly, in Madin–Darby Canine Kidney (MDCK) cells, fodrin is predominantly cytoplasmic until a confluent polarized monolayer is established, suggesting that the linkage of fodrin to the membrane is dynamically regulated during cell maturation.[10,22]

In the present study, the distribution and abundance of fodrin has been examined in 18 colonic neoplasms and four cases of Crohn's disease, and the results compared with normal developmental changes in fodrin along the crypt-to-villus axis and with the changes that occur during differentiation of MDCK cells in culture. In 12 cases, the distribution of villin was also determined. Neoplastic enterocytes displayed increased concentrations of fodrin in the cytoplasm and in the apical domain. Increased steady-state levels of fodrin were not observed in the regenerating or quiescent epithelium of Crohn's disease patients, although these patients did have increased apical concentrations of fodrin. In two cases of ductal carcinoma of the breast, increased fodrin immunoreactivity was also noted in the neoplastic cells. Collectively, these results indicate that while fodrin is not a tissue-specific marker, its distribution and abundance may be a sensitive marker of the phenotypic changes that accompany certain pathologic processes in epithelial cells, and that enhanced fodrin levels may be a general feature of both benign and malignant neoplasia.

Materials and Methods

All chemicals were of reagent grade and, unless otherwise indicated, were purchased from Sigma Chemical Corp. (St. Louis, MO) or from Bio-Rad Laboratories (Richmond, CA). The total protein in tissue samples was measured as the OD_{280} of sodium dodecyl sulfate (SDS) solubilized samples, and it was assumed that 1 mg/ml of protein corresponded to 1 optical density (OD) unit. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli.[25] Staphylococcal protein A was labeled with [^{125}I] (Amersham Corp., Arlington Heights, IL) using Iodogen (Pierce Chemical Co., Rockville, IL). Proteins were detected by Coomassie blue staining. Autoradiography was done at −70 C using XAR film (Kodak, Rochester, MN) and fluorescent intensifying screens. Quantitative densitometry of the autoradiograms was performed using a Visage 2000 analytical imaging instrument (BioImage Inc., Ann Arbor, MI). All procedures were carried out at room temperature, unless otherwise indicated.

Tissue and Cells

All tissue samples were obtained from specimens submitted to the Pathology Department after their removal at Yale–New Haven Hospital. Specimens were secured within 30 minutes of their resection, under the auspices of a Yale Human Investigation Protocol. With the supervision of the responsible pathologist, bowel specimens were opened immediately after resection, rinsed with cold (4 C) isotonic saline, and photographed. Full-thickness sections of a tumor edge that also contained grossly normal mucosa, and areas remote from the tumor, which appeared to be uninvolved, were fixed overnight in acetone, cleared in xylene for 90 minutes, infiltrated with paraffin at 60 C for 90 minutes, and embedded. For the cases of Crohn's disease, full-thickness sections were taken from the colonic portion of four resection specimens that contained both small bowel and colon. Ductal adenocarcinomas of the breast were obtained from material that remained after routine frozen section examination; it was fixed and prepared as above. Colon samples were also frozen in liquid N_2 and stored at −70 C until used for the quantitative immunoblotting studies. MDCK cells (ATCC-MDCK-CCL34), originally obtained from the American Type Culture Collection (Rockville, MD), were subcloned and maintained in modified Eagles' minimal essential medium with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Trypsinized cells were cultured on collagen-coated glass coverslips and plated at different densities to obtain isolated (contact naive) cells or confluent monolayers.[11]

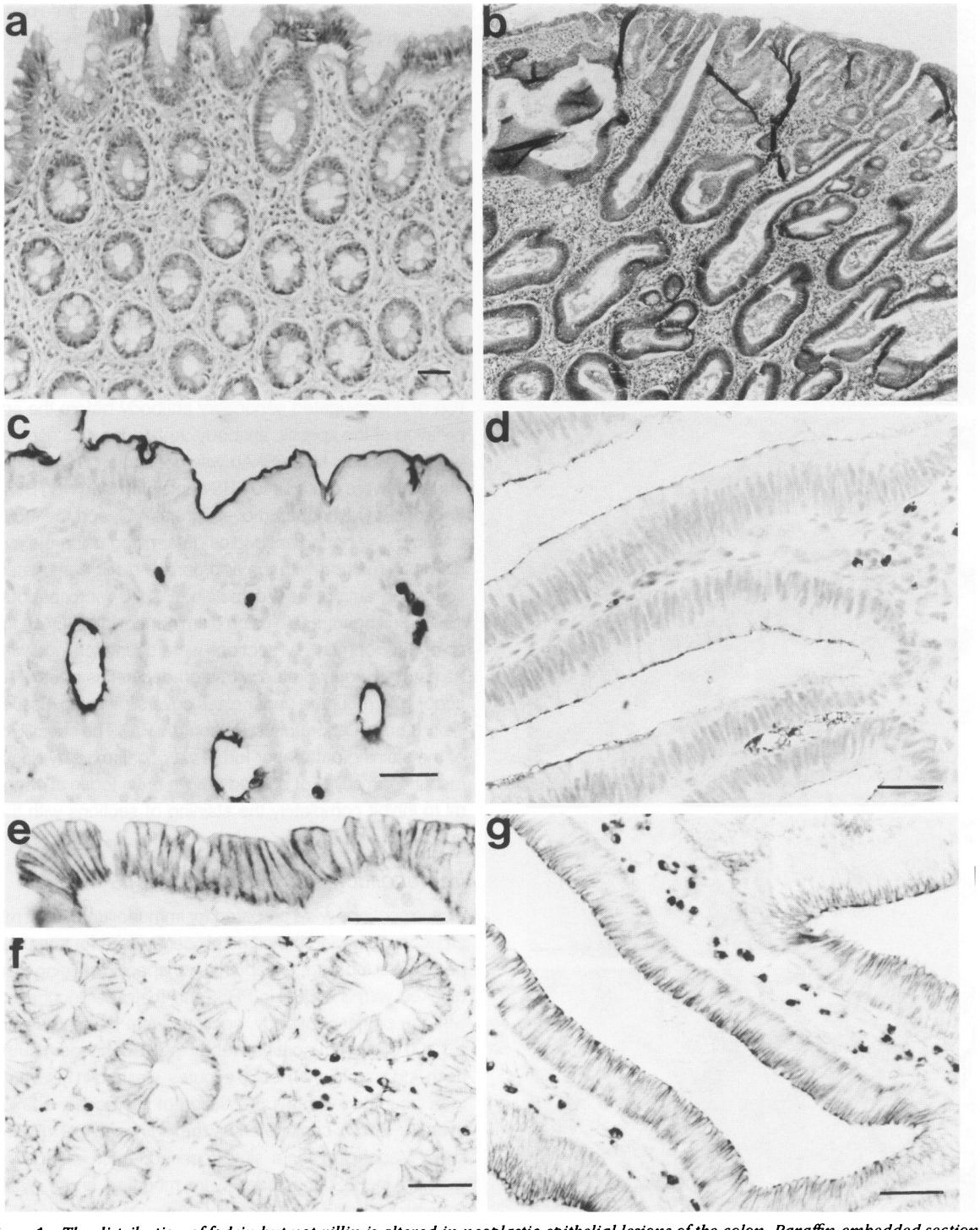

Figure 1. *The distribution of fodrin but not villin is altered in neoplastic epithelial lesions of the colon. Paraffin-embedded sections of acetone-fixed normal human colon* (a, c, e, f) *or an adenomatous polyp* (b, d, g) *were stained with hematoxylin and eosin* (a, b), *or by immunoperoxidase using anti-villin Mab23kAB* (c, d), *or polyclonal anti-fodrin antibody* (e, f, g). *Note that while villin is always confined to the apical (lumenal) border of enterocytes, fodrin only appears there during normal crypt-to-villus development when the cells reach the surface. In adenomatous polyps, fodrin is found at the apical domain of the cells throughout the lesion. The sections shown in* c *and* d *are counterstained with hemotoxylin, (bar = 50 μm).*

Antibodies

The preparation and characterization of the rabbit anti-human fodrin antibody has been described.[26] This antibody reacts primarily with the α-subunit of fodrin and demonstrates broad species cross-reactivity. It was affinity purified on a human fodrin-sepharose CL-4B (Pharmacia, Uppsala, Sweden) affinity column. The mono-

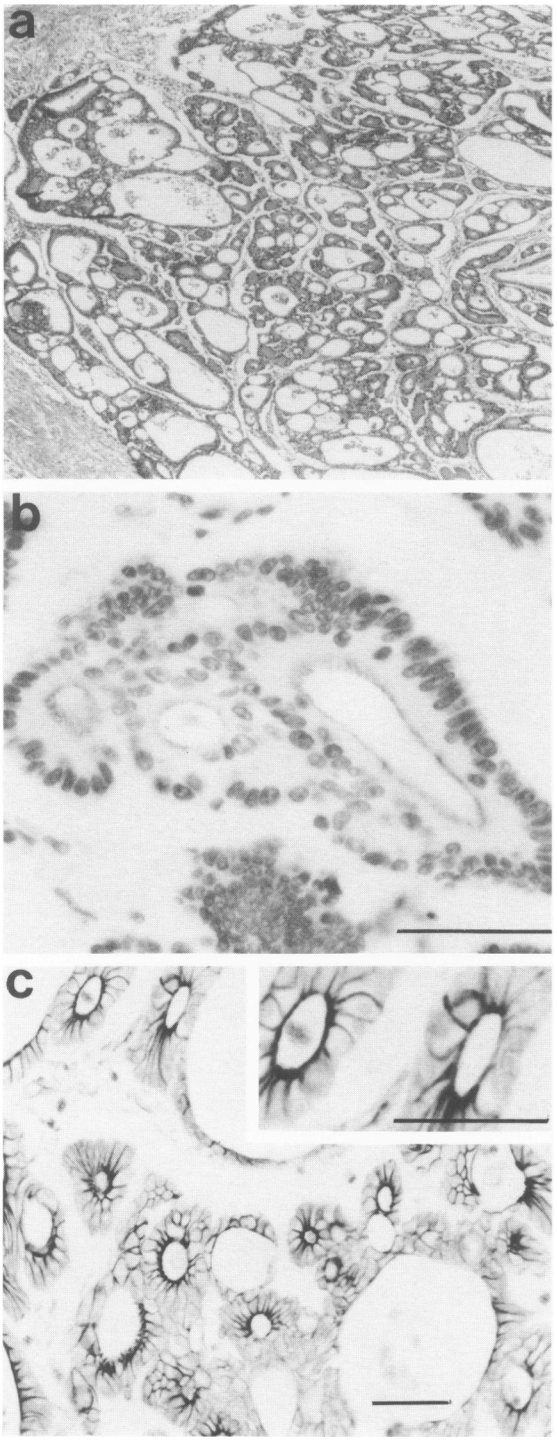

Figure 2. *Adenocarcinomas of the colon demonstrate marked accumulation of fodrin in an apical and cytoplasmic location. Paraffin-embedded sections of acetone-fixed specimens from invasive adenocarcinomas of the colon were stained with hematoxylin and eosin* (a), *immunoperoxidase using anti-villin Mab23kAB* (b), *or with polyclonal anti-fodrin antibody* (c). *Note that while the distribution of villin was unchanged (in most lesions, see Table 1), there was intense staining of fodrin at the apical domain of the cells throughout the lesion and increased cytoplasmic abundance of this protein as well. The changes seen in the adenocarcinomas were always greater than those seen in the adenomas (Figure 1). The apparent staining of the nuclei in* b *arises from the hemotoxylin staining of the nuclei because these photomicrographs were taken without a blue filter. There is no staining of the nucleus with the anti-villin antibody (bar = 50 μm).*

clonal antibody to human villin (Mab23kAB) has also been characterized previously.[6]

Immunoperoxidase Staining of Tissue Sections

Five μm-thick sections were prepared from paraffin-embedded tissue blocks using standard methods[5] and floated onto glass slides previously coated by immersion with 0.1 mg/ml poly-L-lysine. The paraffin was melted by incubating the slides at 60 C for 2 hours, and then deparaffinized by three washes in xylene, followed by two washes in acetone. All washes were for five minutes each. Before immunostaining, the sections were rehydrated for ten minutes in PBS. Nonspecific staining was blocked by incubation of the sections for 30 minutes with a 1:20 dilution of unconjugated secondary antibody before the application of the specific antibody. Anti-fodrin was used at a 1:500 dilution, Mab 23kAb was used at a 1:10 dilution, and both were diluted into PBS containing 3% (w/v) BSA. Sections were incubated overnight at 4 C, and the localization of the bound antibodies determined using the avidin-biotin method with the appropriate biotin conjugated secondary antibodies (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) using diaminobenzidine (DAB) for color development. All sections were counterstained with hematoxylin, dehydrated with a graded series of ethyl alcohol, and mounted under glass coverslips using Tissue-Texx (Lerner Laboratories, Pittsburgh, PA). The specificity of the staining pattern for fodrin was confirmed by documenting the ability of purified bovine brain fodrin at 1 mg/ml to inhibit completely the staining of sections with the anti-fodrin antibody.

Immunofluorescent Measurements

Tissue sections were prepared for immunofluorescent microscopy following the same procedures as for immunoperoxidase labeling, except that nonspecific staining was blocked with 3% BSA in PBS, and that after incubation with the primary antibody, sections were incubated with a 1:100 dilution of goat anti-rabbit IgG (Boehringer-Mannheim, Indianapolis, IN) for 30 minutes, washed in PBS, and then incubated with a 1:50 dilution of fluorescein isothiocyanate (FITC) conjugated swine anti-goat IgG (Boehringer-Mannheim) for a similar period. After exhaustive washing in PBS, the slides were mounted under glass coverslips using Aqua-Mount (Lerner Laboratories). MDCK cells were fixed in 4% (w/v) paraformaldehyde in PBS for 30 minutes and then permeabilized for ten minutes in 0.5% (v/v) Triton X-100 in PBS containing 2 mM $MgCl_2$. For the immunofluorescent measurements, affinity-purified anti-fodrin antibody was used at a 1:100 dilu-

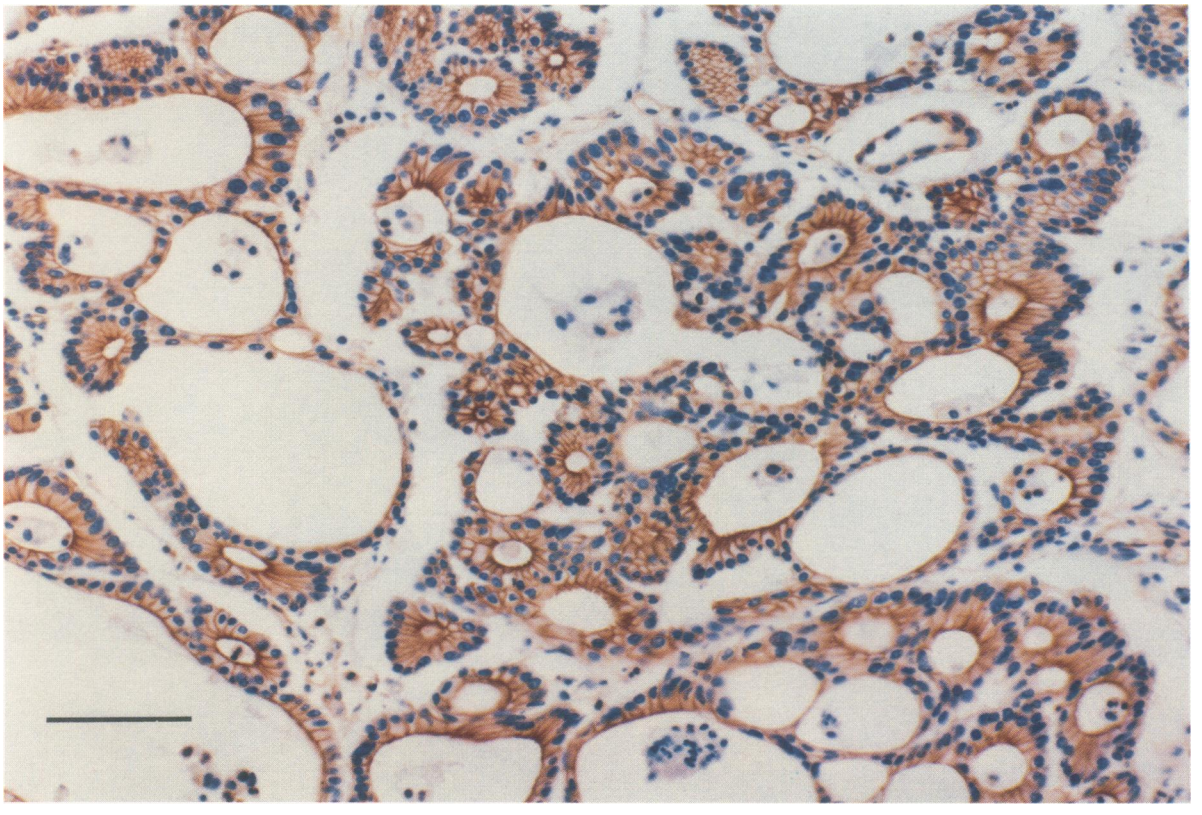

Figure 3. *Abundant cytoplasmic fodrin is a constant feature of colonic adenocarcinomas. The distribution of fodrin in acetone-fixed, paraffin-embedded sections was examined by immunoperoxidase labeling as described in Figure 2. The intense apical and cytoplasmic staining is apparent throughout the lesion (bar = 80 μm).*

tion. The fluorescently labeled sections were visualized using a Zeiss Axiomat microscope equipped for epillumination (Carl Zeiss, West Germany). Alternatively, a Bio-Rad MRC-500 laser confocal imaging system linked to a Zeiss microscope was used to obtain digitized images of 0.3 to 0.6 μm optical planes through the tissue sections. The fluorescent intensity of different cells, and regions within a cell, were compared using the standard data analysis software supplied with the MRC-500 system. All comparisons were made at similar exposure and illumination times to avoid problems with photobleaching of the sample. Neoplastic and histologically normal fields were al-

Table 1. *Distribution of Fodrin and Villin in Various Conditions**

Cell type	Number of cases	Number with staining detected		
		Apical (%)	Basolateral (%)	Cytoplasmic (%)
For Villin				
Normal crypt	12	12 (100)	0 (0)	0 (0)
Adenoma	6	6 (100)	0 (0)	0 (0)
Carcinoma	6	6 (100)	1 (16)	2 (33)
Well differentiated	3	3 (100)	1 (33)	0 (0)
Moderately differentiated	2	2 (100)	1 (50)	1 (50)
Poorly differentiated	1	1 (100)	0 (0)	0 (0)
For Fodrin				
Normal crypt	12	0 (0)†	12 (100)	0 (0)
Adenoma	7	7 (100)	7 (100)	7 (100)
Carcinoma	11	11 (100)	11 (100)	11 (100)
Well differentiated	3	3 (100)	3 (100)	3 (100)
Moderately differentiated	6	6 (100)	6 (100)	6 (100)
Poorly differentiated	2	2 (100)	2 (100)	2 (100)

* A case was judged positive if in three nonoverlapping fields more than 10% of the enterocytes displayed immunoperoxidase labeling in the regions specified.

† Rarely was staining in this region detected, but the intensity of this signal was low.

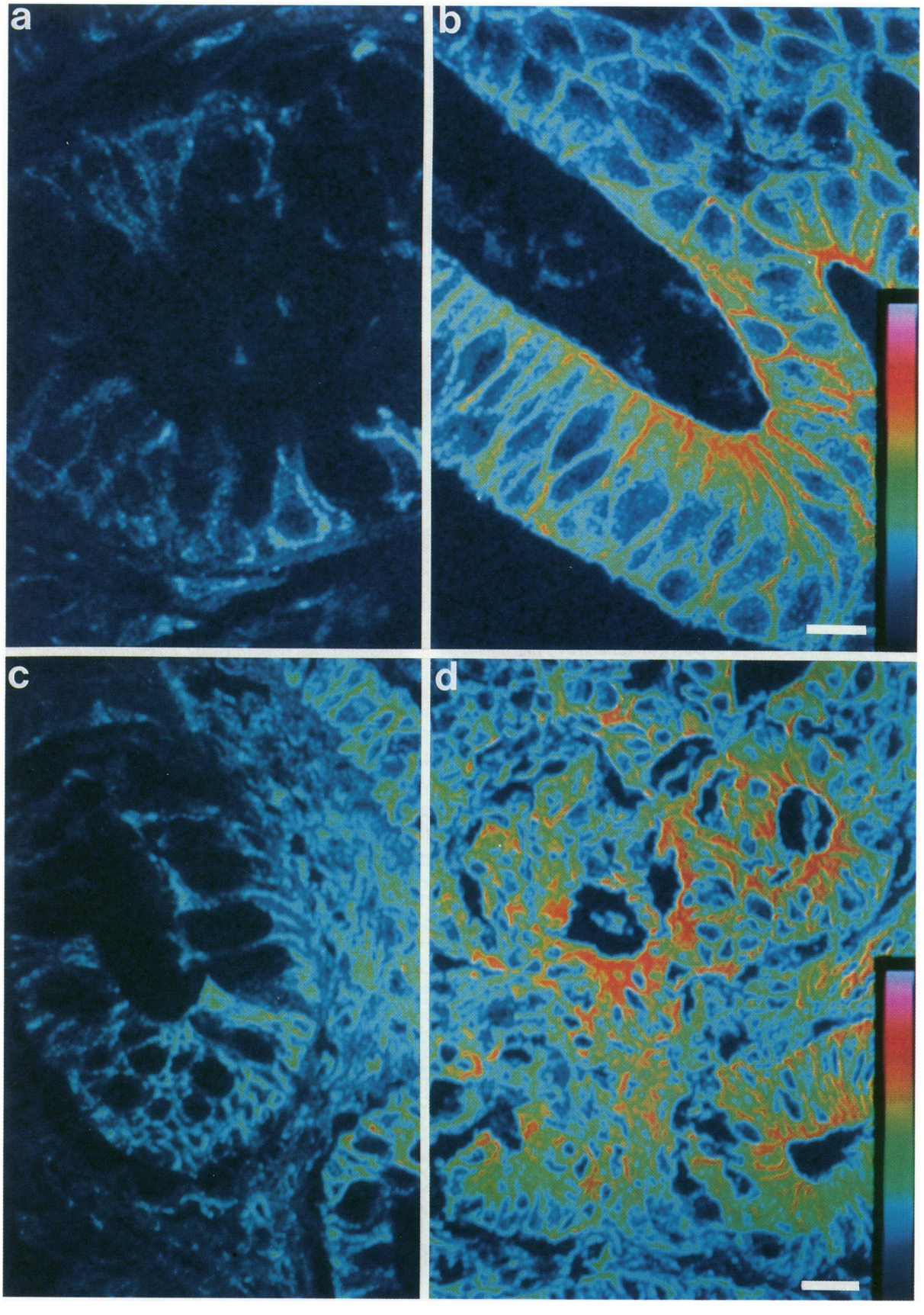
a
b
c
d

Figure 4. *Digital confocal fluorescent microscopy demonstrates the increased abundance of fodrin in adenocarcinomas. Fluorescein-stained sections similar to those shown in Figure 3 were examined using confocal microscopy; the levels of fluorescent intensity are presented as pseudocolored images. The highest levels of staining were set to white and background staining intensity was set to dark blue. Precautions were taken to assure consistent calibration of the instrument, and separate evaluations have determined the linearity of the instrumental response over the range of intensities examined. The results of two separate experiments are presented; paired normal, adenocarcinoma samples are presented in each row: normal colonic crypt enterocytes* (a, c); *invasive colonic adenocarcinoma* (b, d). *The paired images in each row were from separate fields of the same slide and were recorded sequentially with no change in instrumental gain, calibration, or magnification (bar = 30 μm).*

ways compared from different areas of the same section, on the same slide, to avoid any differences that might arise from variations in fixation or processing.

Quantitative Immunoblotting

Frozen tissue (approximately 0.3 to 0.5 g) was finely minced at 0 C and suspended in 7.5 ml of 5% trichloroacetic acid. Solubilized DNA was removed by discarding the supernatant of a five-minute 5000*g* spin at 4 C. The pellet was suspended in 5 to 10 volumes of distilled water and dissolved in an equal volume of 2× SDS-PAGE solubilizing buffer containing 0.5 M 2-mercaptoethanol. Total protein in the solubilized sample was determined spectrophotometrically, after which a trace amount of bromphenol blue was added. The sample was neutralized with ammonium hydroxide vapor, boiled for five minutes, and then analyzed by SDS-PAGE. Proteins were transferred electrophoretically overnight at 30 V to Immobilon membranes (Millipore Corp., Waltham, MA) at 4 C in 12.5 mM Tris, 96 mM glycine, 0.05% (w/v) SDS, pH 8.3, and processed for Western blotting with [^{125}I]-labeled Staphylococcal protein A. The abundance of fodrin in the blots was measured by

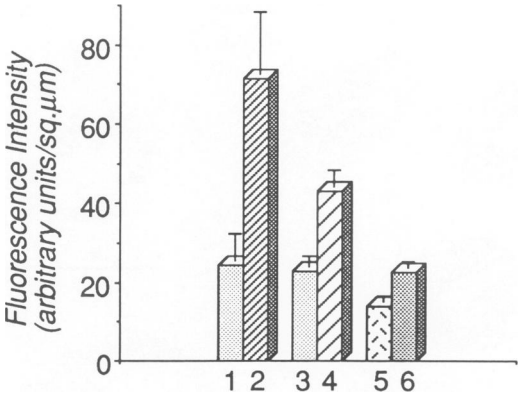

Figure 5. *The amount of fodrin per equivalent areas of epithelium is increased more than threefold in adenocarcinomas. The intensity of fluorescent signal from equal areas of epithelial cells was compared using images similar to those shown in Figure 4. In each case the integrated fluorescent signal from normal epithelium (columns 1, 3) was compared with adjacent areas of adenocarcinoma (column 2) or adenoma (column 4). For the Crohn's disease cases, areas of histologically quiescent glands (column 5) were compared with nearby regenerative glands (column 6). Note the marked increase in fodrin in cancer cells, with lesser degrees of increase in adenomas (error = 1 SEM).*

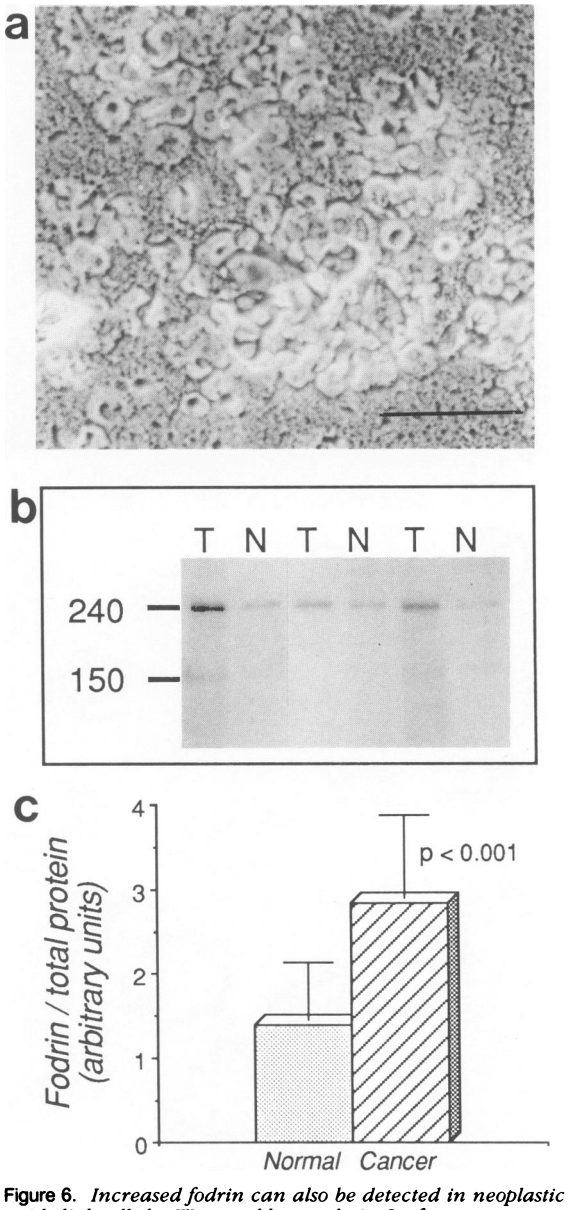

Figure 6. *Increased fodrin can also be detected in neoplastic epithelial cells by Western blot analysis. Surface enterocytes were dissected from normal epithelium or from the surface of adenocarcinomas, homogenized, precipitated in TCA, and then analyzed by SDS-PAGE and Western blotting with anti-fodrin antibodies.* a: *Phase-contrast micrograph of a typical preparation of surface enterocytes used for the blotting experiments (bar = 50 μm).* b: *of the Western blot analysis of samples from three patients. The positions of α-fodrin (240 kDa) and its major proteolytic product at 150 kDa are indicated. Nearly equivalent amounts (on a total protein basis) of tumor (T) or normal (N) tissue was loaded in each case.* c: *Quantitative comparison of total fodrin per total cellular protein, as measured by densitometry of the autoradiogram shown in* b. *Note the more than twofold enhancement of fodrin per total cellular protein in the adenocarcinomas (n = 4; error bar = 1 SEM).*

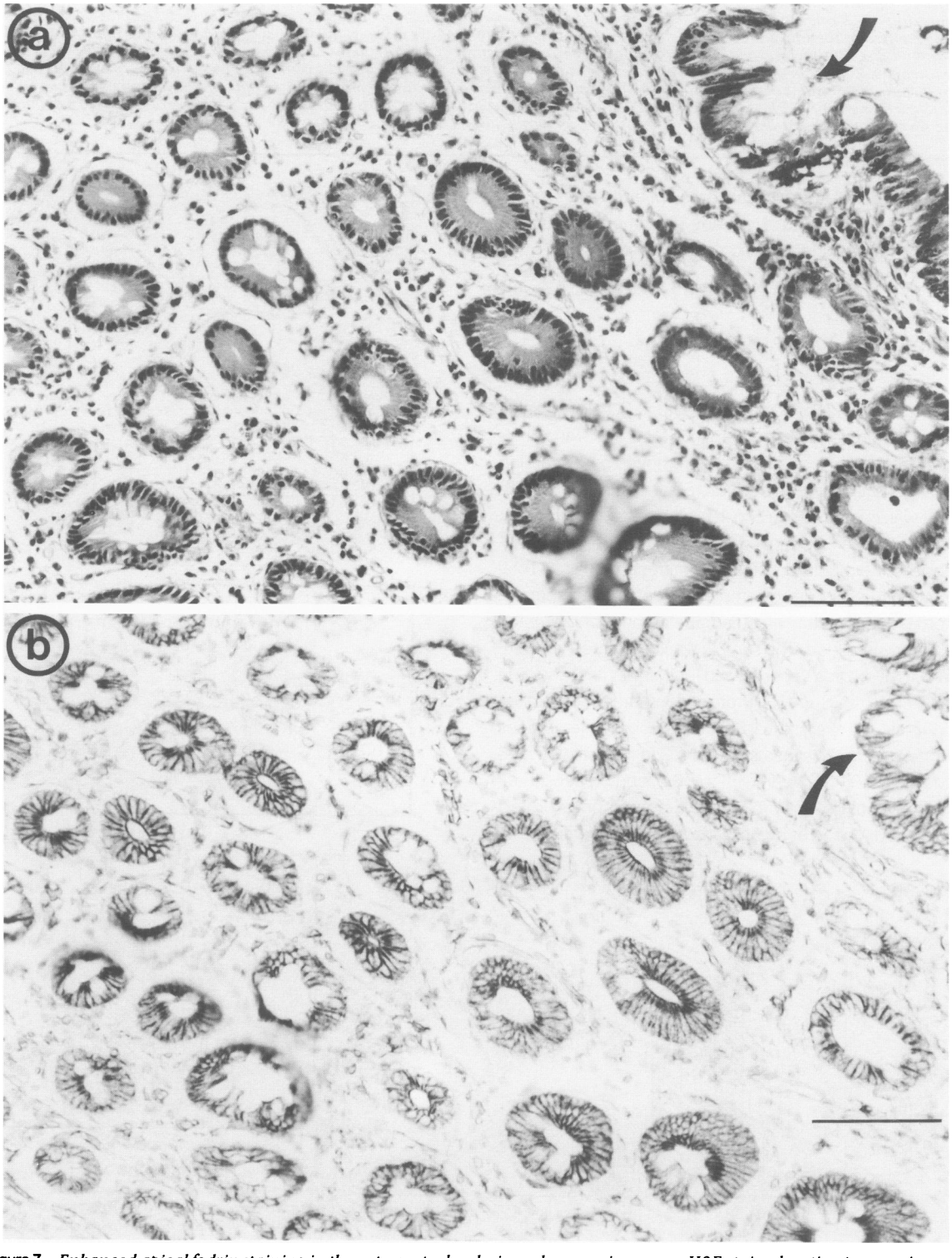

Figure 7. *Enhanced apical fodrin staining in the enterocytes bordering adenocarcinomas.* **a:** *H&E-stained section from region adjacent to invasive adenocarcinoma. The area of tumor is marked by an arrow.* **b:** *A consecutive section stained with anti-fodrin antibodies, immunoperoxidase technique. Note the dense ring of staining about the lumen of the crypts nearest the tumor (arrow) (bar = 100 μm).*

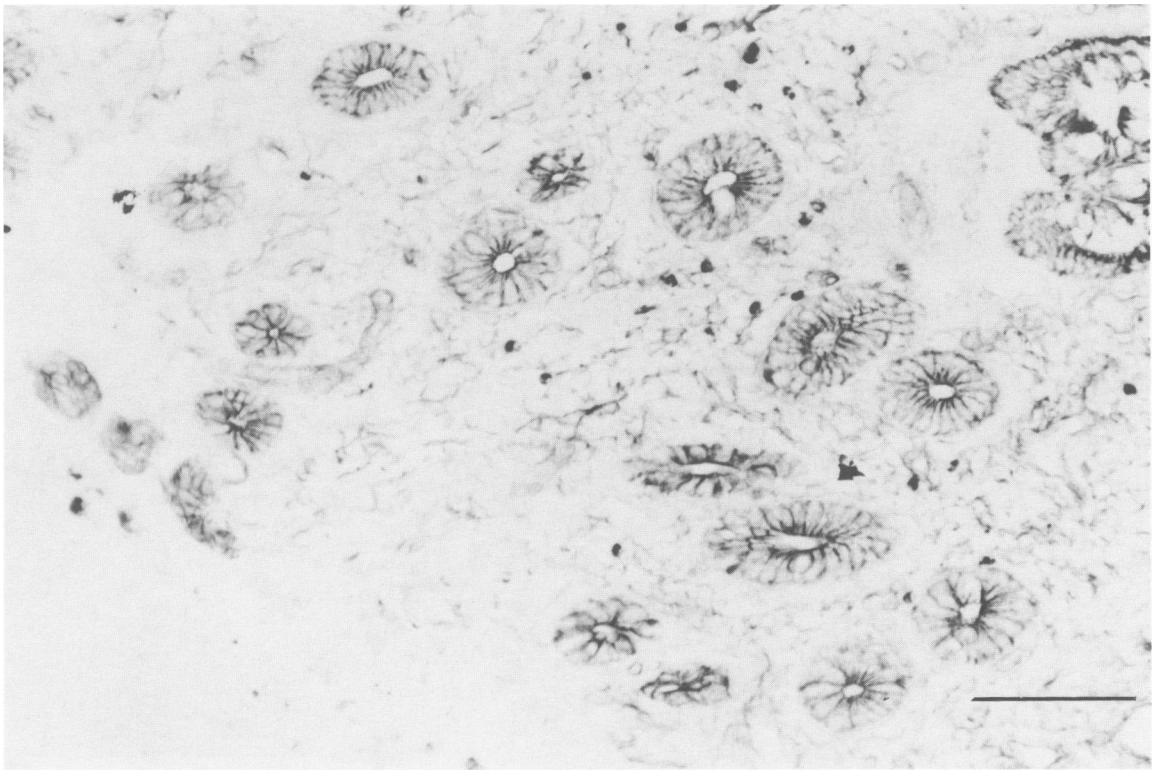

Figure 8. *Enhanced apical fodrin in Crohn's disease. An area of ascending colon involved with Crohn's disease was stained with antibodies to fodrin, immunoperoxidase technique. Note the dark ring of staining about the lumens of the crypts (bar = 100 μm).*

densitometry of the autoradiograms, followed by a comparison with Western blots of standard amounts of purified bovine brain fodrin. All values were normalized to equal amounts of total protein loaded onto the gels.

Results

The Normal Differential Assembly of Villin and Fodrin at the Apical Domain of the Enterocyte Is Not Found in Neoplastic Lesions

During normal crypt-to-villus maturation, enterocytes destined to become absorptive and mucin-secreting cells migrate from a proliferative zone near the base of the crypt to the tip of the microvillus. In the normal human colon (Figure 1a), the quantity of villin increases as the cells mature, but in all enterocytes it is confined almost exclusively to their apical domain (Figure 1c). This is true for both the cells in the crypts, as well as those at the lumenal surface. In contrast, in crypt enterocytes, immunoreactive fodrin is largely confined to the basolateral domain, with only sparse staining of the apical portions of these cells (Figure 1f). This distribution persists until the zone of cells closest to the lumen, in which intense apical staining of fodrin abruptly becomes apparent (Figure 1e). Presumably, this increased apical fodrin staining reflects its assembly into the prominent terminal web (TW) of the mature brush-border cytoskeleton. This pattern is confirmed by the quantitative immunofluorescent measurements shown below. Significantly, throughout the normal differentiation process, cytoplasmic fodrin is rarely evident.

In neoplastic lesions (Figure 1b, 2a) of the colon, the pattern of fodrin staining, but not villin, is consistently altered (Figures 1d, g; 2b, c). In all of the adenomas that were examined, increased cytoplasmic and apical staining for fodrin was apparent (Figures 2c, 3). No differences were seen in the staining pattern of the neoplastic cells as a function of their location, regardless of whether they were deep in the lesion or near the surface. The apical fodrin staining of adenomas, although prominent, rarely was confluent enough to form a continuous dark ring about the lumen in transverse sections of the crypt. In adenocarcinomas, however, continuous lumenal staining was characteristic (Figures 2c, 3). In these lesions, representing tumors of various grades, the malignant cells stained more strongly for fodrin than those seen in either normal or adenomatous mucosa; cytoplasmic and apical staining was especially marked in these lesions. This was true even in glands with unapparent cellular crowding and minimal cytologic atypia.

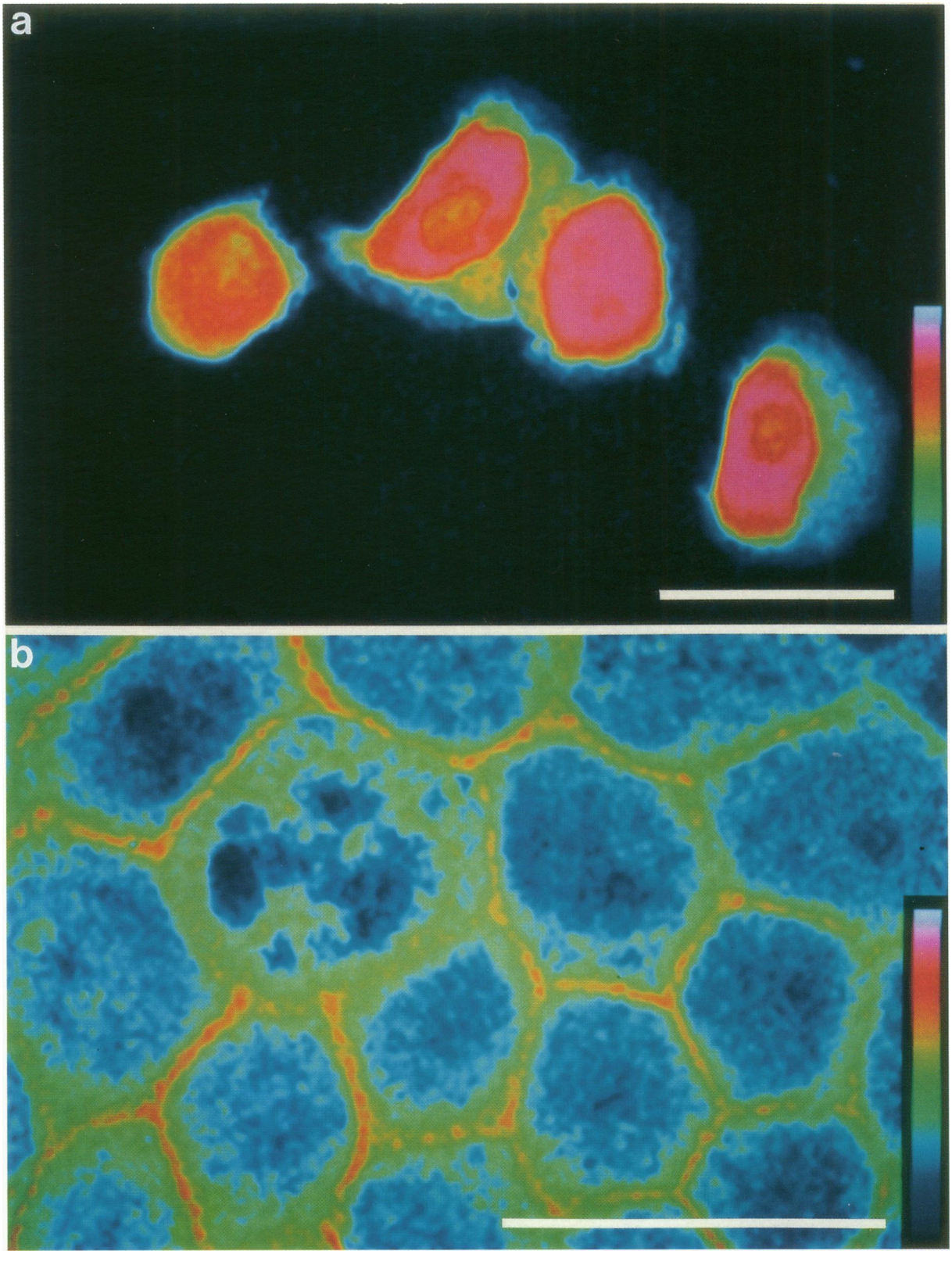

Figure 9. *Nonconfluent MDCK cells display high levels of cytoplasmic fodrin. MDCK cells were grown in cell culture, and the distribution and abundance of fodrin was measured by integration of the fodrin signal using digital confical microscopy.* **a:** *Isolated MDCK cells, which lack polarized features, display high levels of cytoplasmic fodrin and have no detectable fodrin concentrated at the plasma membrane.* **b:** *Confluent MDCK cells form an organized monolayer that is highly polarized. In these cells, the distribution of fodrin is almost exclusively basolateral. The total level of fodrin per cell also decreases by about fourfold in the confluent monolayers. Shown are pseudocolored confocal microscope images (bar = 30 μm).*

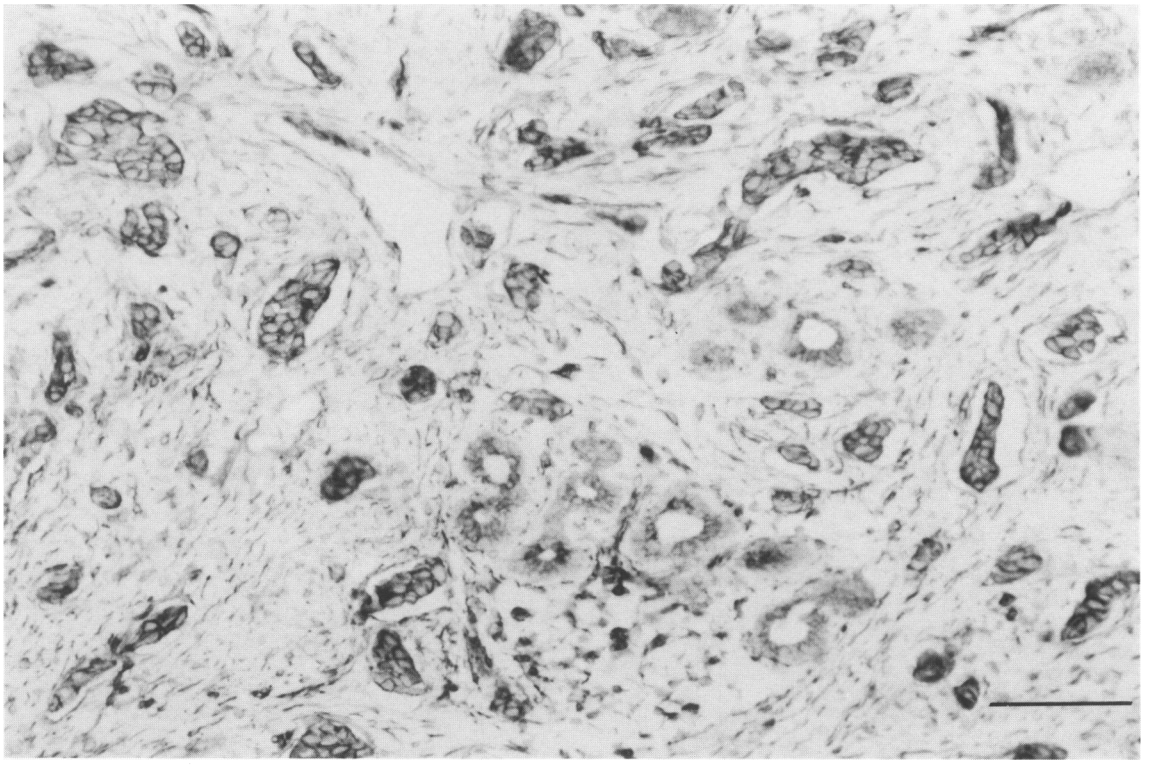

Figure 10. *Fodrin immunoreactivity is increased in breast carcinoma. An acetone-fixed, paraffin-embedded section of invasive ductal carcinoma of the breast was stained with anti-fodrin antibodies, immunoperoxidase technique. A normal lobular unit is visible in the center of the field, faintly stained. The intense staining of the surrounding carcinoma is evident (bar = 100 μm).*

The distribution of villin was also examined in six adenomas (Figure 1d) and in six adenocarcinomas (Figure 2b). Generally, in all of these lesions there was prominent staining of the apical membrane, except in one lesion in which villin staining was lost from the cells in regions of the tumor that were the most anaplastic. In addition, as previously noted,[6] cytoplasmic villin staining was found in two carcinomas and basal membrane staining was found in one (Table 1).

The Total Amount of Enterocyte Fodrin Is Increased Threefold in Colonic Adenocarcinomas

The results of the immunoperoxidase studies suggested not only that there was a redistribution of fodrin in transformed cells, but also that the total amount of fodrin in each cell was increased. Quantitative immunofluorescent confocal microscopy was used to further examine these conclusions. With this technique, the fluorescent intensity that arises from a thin (<1 μm) optical plane of uniform thickness through a sample can be quantitatively examined as a function of its distribution within the cell. Conventional fluorescent microscopy confirmed the qualitative conclusions reached with immunoperoxidase labeling. Using the confocal microscope, the enhanced apical distribution of fodrin in adenomas and carcinomas was particularly apparent. A pseudocolor confocal microscope image is shown in Figure 4. In these images, the intensity of the fluorescence is marked by the color. Normal crypt enterocytes displayed relatively low levels of fodrin, distributed almost exclusively along the basolateral aspects of the cell (Figure 4a and c). In separate regions (from the same slides), which contained an adenocarcinoma, there was a marked increase in the abundance of fodrin in each cell and an increase in the amount distributed in apical and cytoplasmic pools (Figure 4b and d).

To obtain a quantitative estimate of the change in total fodrin per cell in these lesions, the fluorescent intensity arising from equivalent areas of epithelial cells was digitally integrated from normal or neoplastic regions of the same slide (Figure 5). This analysis indicated that during normal crypt-to-villus maturation the total level of fodrin/enterocyte increased about 1.7-fold. When normal enterocytes (crypt) (Figure 5, columns 1, 3) were compared with adenomas (column 4), the adenomas were found to contain 1.9 ± 0.4 times as much fodrin as adjacent normal-appearing enterocytes. In adenocarcinomas (column 2), this effect was even more prominent, with malignant lesions displaying a 2.9 ± 1.5-fold increase in enterocyte levels of fodrin. By way of comparison, the level of fodrin was also measured in the regenerating and quiescent epithelium of bowel removed from three pa-

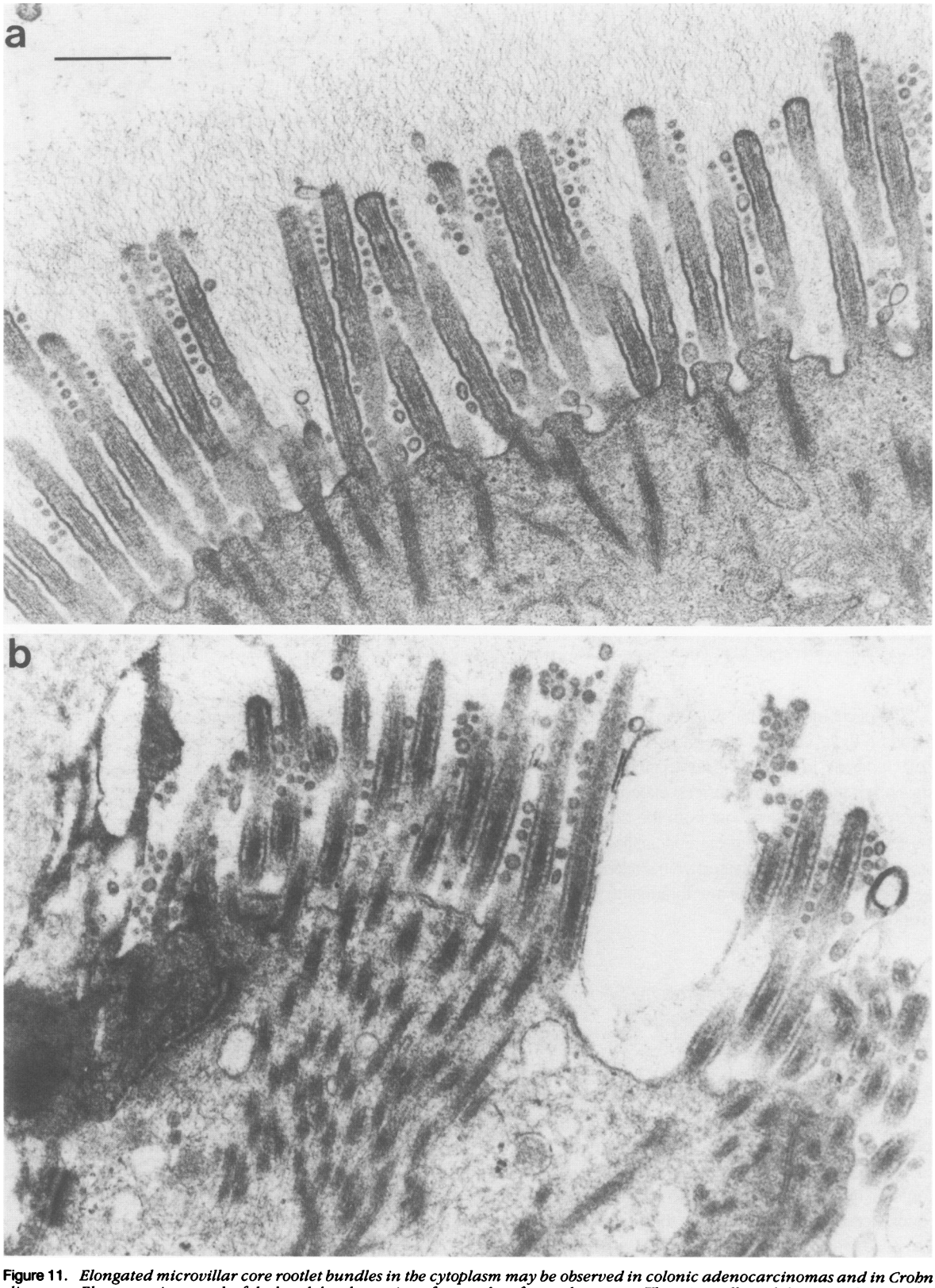

Figure 11. *Elongated microvillar core rootlet bundles in the cytoplasm may be observed in colonic adenocarcinomas and in Crohn's disease.* **a:** *Electron micrograph of the brush-border region of normal surface colonocytes. The microvilli and the central microvillar core rootlet (actin) bundles are apparent. The exclusion of organelles in the vicinity of the apical membrane is thought to be due to the terminal web that forms in this region.* **b:** *Electron micrograph of an enterocyte from a region of colon involved with Crohn's disease.* **c:** *Electron micrograph of the apical domain of an enterocyte from a well-differentiated colon adenocarcinoma. Note the prominent extensions of the microvillar actin bundles into the cytoplasm in both the Crohn's sample and particularly in the adenocarcinoma. There is also marked blunting of the microvilli in the adenocarcinoma sample (glutaraldehyde fixed, postfixed in osmium tetroxide; bar = 0.64 μm).*

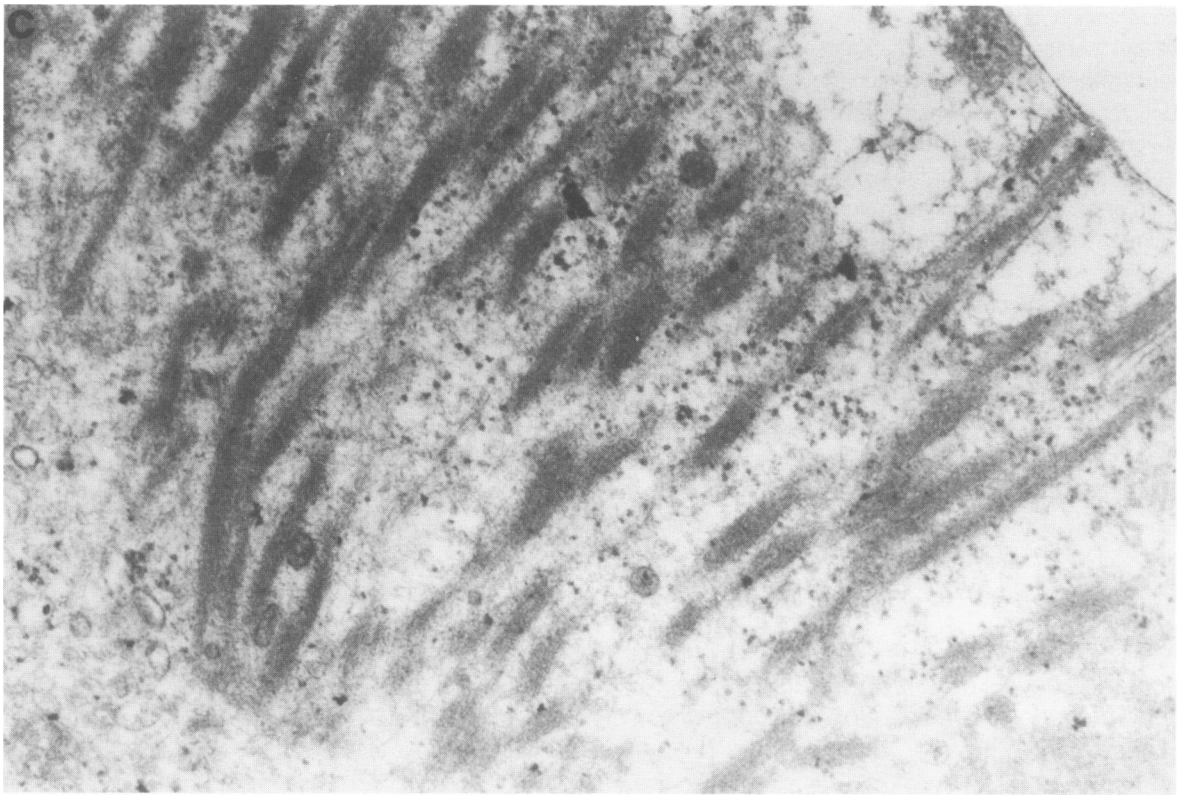

Figure 11 (*continued*)

tients with Crohn's disease (Figure 5). In these patients, the overall levels of fodrin did not appear to significantly exceed those of normal enterocytes, and the most actively regenerating areas averaged only a 1.6 ± 0.3-fold enhancement in enterocyte fodrin compared to the adjacent quiescent areas.

Finally, it was desirable to quantitate the level of fodrin in intestinal mucosa by an alternative method to verify the results obtained by digital microscopy. This was done by quantitative Western blotting of equal-sized samples of intestinal epithelium dissected from the frozen tissue. Phase-contrast microscopy was used to assure that the cellular material taken for these analyses consisted predominantly of epithelial cells (Figure 6a). Three cases were analyzed in this fashion; autoradiograms of their [^{125}I]-labeled Western blots are shown in Figure 6b. In each case, the tumor contained significantly more fodrin relative to total cellular protein than did the control tissue ($P < 0.001$ by the paired Student's *t*-test; Figure 6c). The average increase was 2.0 ± 1.3, a value similar to the results obtained by digital microscopy.

Enterocytes in Crohn's Disease and Those at the Border of Tumors Display Increased Apical Fodrin

An increase in the total fodrin per cell and the presence of significant cytoplasmic pools of fodrin appeared to be characteristic of adenomas and adenocarcinomas. Conversely, the intense apical redistribution of fodrin, also a feature of these lesions, was found to be present in other settings as well. This was occasionally most noticeable in the cytologically normal glands immediately adjacent to an adenocarcinoma (Figure 7). Most enterocytes in these border zone regions displayed a dark ring of lumenal fodrin staining, even though by other morphologic criteria they appeared to be normal. Similar changes were also seen in the regenerating colonic epithelium of specimens removed for Crohn's disease (Figure 8).

The Cellular Content of Fodrin Is Enhanced in Undifferentiated Epithelial Cells in Culture

To better understand the changes in fodrin distribution and abundance that occur during epithelial cell maturation, advantage was taken of the fact that isolated (undifferentiated) MDCK cells form highly polarized epithelial monolayers when grown to confluence in cell culture. Although these cells do not form an elaborate apical brush border, they do display marked changes in their fodrin distribution and abundance as a function of development (Figure 9). Isolated, unpolarized MDCK cells plated at sparse density on either plastic or collagen substrates are characterized by a relatively high and diffuse cytoplasmic

concentration of fodrin (Figure 9a).[10,22] Coincident with cell–cell contact and the establishment of a polarized monolayer, the total fodrin content of these cells falls by about fourfold as determined by quantitative confocal microscopy (as in Figure 5), and the protein redistributes to the basolateral margin of the cell (Figure 9b). Thus the fodrin distribution in confluent monolayers of MDCK cells is similar to that of crypt enterocytes. Similarly, the cytoplasmic fodrin distribution found in unpolarized MDCK cells resembles the changes seen in neoplastic lesions, perhaps not a surprising result for an immortal cell line. However, it is important to note that this analogy is not exact because in confluent MDCK cells fodrin does sort to the basolateral margins of the cell, while in tumor cells *in vivo,* even though these cells establish cell contacts, a significant fraction of the fodrin remains cytoplasmic. The failure of fodrin to assemble at the apical domain in the MDCK cells probably reflects their lack of a well-developed brush border.

Increased Levels of Fodrin Are Also Characteristic of Other Epithelial Neoplasms

Other common epithelial tumors were examined in acetone-fixed tissue sections for evidence of increased fodrin immunoreactivity by the immunoperoxidase technique. These included two adenocarcinomas of the breast, two adenocarcinomas of the stomach, one adenocarcinoma of the esophagus, and one adenocarcinoma of the small intestine; all demonstrated such a change. One of the breast lesions is shown in Figure 10, where the cancerous cells can be easily distinguished from normal glands by the intensity of fodrin staining alone.

Discussion

The studies reported here document differential rates of assembly of villin and fodrin at the apical domain of enterocytes during normal crypt-to-villus maturation in the human colon, and describe two nonexclusive aberrations in fodrin dynamics that may characterize two classes of pathologic change: 1) a redistribution of fodrin to the apical domain, and 2) increased total cellular fodrin with diffuse cytoplasmic staining. The former alteration appears to accompany both reactive and neoplastic lesions afflicting enterocytes, while the latter change has so far only been found in neoplastic lesions of both enterocytes and other epithelial cells. Villin, which was also examined, did not undergo such dramatic shifts, as noted before.[5,6,16,17]

Several studies have examined the assembly of the intestinal brush border and its associated proteins both during embryogenesis and normal crypt-to-villus maturation. In the chicken, both processes appear to be similar. First blunt microvilli appear, followed by the elongation of the microvillar core actin rootlets, formation of the spectrin-containing terminal web, and extension of the microvilli.[13,27] At the molecular level, each protein in the chicken appears to be expressed and assembled into the brush border in a sequential fashion: villin first, followed by fimbrin and brush border myosin I (110K-CM). Chicken brush-border spectrin (TW260/240) first appears with the onset of terminal web assembly,[28,29] suggesting that in this creature its assembly is largely regulated at the transcriptional or translational level. The present study (for crypt-to-villus development), as well as other recent reports examining the embryonic development of mammalian enterocytes,[14,15,29] demonstrate that in humans spectrin (fodrin) is present even in the most immature enterocytes but is not assembled into the terminal web until late in the developmental process. Thus in mammals factors other than the rate of total fodrin accumulation in the cell presumably control its assembly into the terminal web. We spectulate that the increased abundance of fodrin at the apical surface of enterocytes in adenomas, carcinomas, Crohn's disease, and border-zone epithelium relates to an increased extension of the microvillar core actin bundles into the cytoplasm, despite the shortening of the microvilli that is generally observed in such processes. Increased exposure of the microvillar cores would be expected to lead to a thickened web, with a correspondingly increased amount of associated fodrin. The observation of elongated microvillar core rootlets in electron photomicrographs of colonic adenocarcinomas and Crohn's disease supports this conjecture (Figure 11). This hypothesis is summarized in Figure 12.

A second significant observation to come from this work is the recognition that elevated levels of fodrin and its cytoplasmic distribution appear to characterize neoplastic epithelium. In studies of cultured MDCK cells, it has been noted that coincident with cell–cell contact, fodrin is stabilized at the basolateral plasma membrane and cytoplasmic pools disappear.[10,22] As reported here, there is also a marked decrease in the abundance of fodrin in MDCK cells during this apparent maturation process. This is in marked contrast to the finding here of increased fodrin levels in mature enterocytes at the villus tip, or of diminished steady-state levels of fodrin in immature Xenopus embryos.[30] Thus the finding of increased fodrin in a cytoplasmic distribution in neoplastic tissues and in transformed cells in culture suggests that this may be a peculiar property by which such cells can be recognized. The genesis of this effect is uncertain, although a global elimination of the constraints that polarize the cell seems unlikely because in most adenocarcinomas an apical distribution of microvilli and villin is usually maintained. Thus while not a tissue-specific marker, increased total and cytoplasmic fodrin may prove useful as a general marker

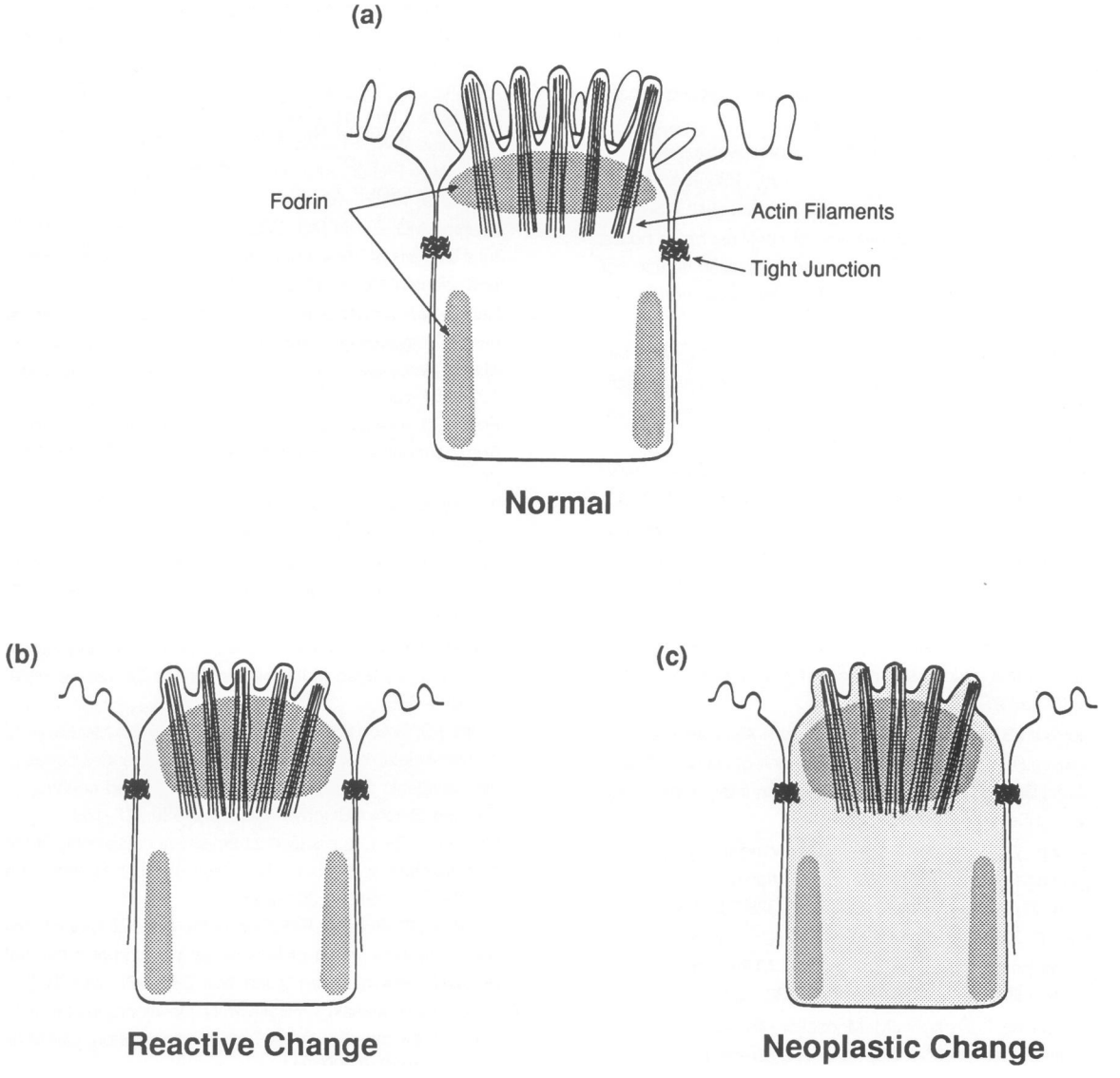

Figure 12. *Hypothesis: the response of fodrin to pathologic stress in enterocytes.* **a:** *Normal mature enterocytes enjoy an exclusively basolateral and apical fodrin distribution and have long, slender microvilli. Normal crypt enterocytes do not assemble fodrin appreciably into their terminal web regions, even though villin and microfilament actin bundles are already present in these cells. The reason that fodrin assembly is delayed during normal crypt-to-tip development is not understood.* **b:** *Under some (degenerative or reactive) pathologic conditions, there is blunting or disruption of the microvilli and an increase in the projection of the microvillar core rootlet bundles into the cytoplasm. We hypothesize that this leads to supranormal accumulation of fodrin in the region of the terminal web in cells competent for the assembly of terminal web fodrin and accounts for the enhanced fodrin staining observed in this region in Crohn's disease and in the enterocytes adjacent to some tumors.* **c:** *With neoplastic transformation there is disruption of normal cytoskeletal targeting mechanisms and the control of fodrin levels within the cell. The result is markedly increased total and cytoplasmic levels of fodrin, a phenomenon so far only observed in neoplastic epithelial cells or in immortalized cells in culture. In neoplastic enterocytes, it is hypothesized that both processes are active (increased terminal web fodrin* and *increased cytoplasmic and total fodrin).*

of neoplastic change. As such it represents a potentially useful tool for distinguishing reactive or regenerative changes from neoplasia, especially in small biopsies or cytologic specimens.

Finally, this study has demonstrated the utility of immunofluorescent digital confocal microscopy to quantitatively evaluate subtle differences between normal and diseased tissue with relative ease, even on paraffin-embedded sections. As such instruments become more widely available, it is likely that quantitative evaluations will play an increasing role in the examination of pathologic tissues stained with existing or future immunohistochemical reagents.

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