Villin—A Marker of Brush Border Differentiation and Cellular Origin in Human Renal Cell Carcinoma

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Expression of villin, a 95-kd F-actin bundling and severing protein, is restricted in animal tissues to epithelial cells with a brush border. Thus, the enterocytes of the intestine and epithelial cells of proximal but not distal tubules of the kidney are strongly positive. Here we report a similar staining pattern for human intestine and kidney. In four human colon adenocarcinomas villin expression was seen in tubular and glandular structures but not in the undifferentiated parts. Fourteen human renal carcinomas (9-Grade I and 5 Grade II) were villin-positive, and 2 sarcomatous renal carcinomas (Grade III) were villin-negative. The percentage of tumor cells that were villin-positive varied from 10–90% for the Grade I and II types. Our results indicate that villin may be a grading marker that deserves further study in renal carcinoma. They also raise the question whether the majority of renal carcinomas are derived from the proximal tubular epithelium rather than from the distal epithelium. (Am J Pathol 1986, 124:294–302)

ULTRASTRUCTURAL studies have recognized microvilli, rudimentary forms of brush border, as well as desmosomes in experimentally induced renal cell carcinomas and both clear and granular cell types of human renal carcinomas.[1-9] Immunohistochemical studies have also shown that antibodies prepared against human kidney microsomes or against a crude brush border fraction stain not only brush borders but also most renal cell carcinomas.[10,11] It was therefore suggested that renal cell carcinomas result from a malignant transformation of proximal tubule cells, because these cells, in contrast to those of the distal tubules, have a brush border with well-developed microvilli.[12,13] This hypothesis has been disputed because the number of specimens studied was small and an antigen thought to be specific for distal tubules could be demonstrated in another investigation of renal carcinoma.[14]

Renal cell carcinomas show a variegate histologic appearance[15]; based on this, multiple grading systems have been devised. Some systems are mainly based on overall structure and cellular morphology,[16,17] while others rely on nuclear morphology.[18,19] Although some grading systems have been shown to correlate reasonably well with survival rate,[16,20,21] they seem to lack easy reproducibility between different observers, so much so that some observers even negate the clinical value of histologic grades for renal cell carcinomas.[13,15] Thus, strictly defined differentiation markers may aid in defining a grading system less dependent on subjective assessment.

Recent biochemical and immunologic studies draw attention to villin, a 95-kd molecular weight protein as a marker of microvilli present on a brush border.[22-26] Some thousand microvilli protrude into the lumen from the apical surface of a polarized epithelial cell such as the enterocyte. This extreme membrane extension is dependent on the core filament bundle underlying each microvillus. The core microfilament bundle can be isolated with intact ultrastructure from chicken intestine. Biochemical analyses revealed that it consists of F-actin and four major associated proteins.[22,25-27] Two of these—villin and fimbrin—have been characterized as F-actin bundling proteins. Antibodies used in histologic study of animal tissues provide an overview of the expression patterns of these two proteins. Whereas fimbrin was recognized as a more general actin binding protein

Supported in part by grants from the Deutsche Forschungsgemeinschaft to Dr. Gröne and from the Deutsche Krebshilfe to Dr. Osborn.

Accepted for publication March 31, 1986.

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present also, for instance, in fibroblasts,[27] villin, first isolated in 1980,[22] showed a much more restricted expression pattern. Although absent from a variety of cell types, villin is abundantly present in the intestinal epithelium[22,25,26] and the renal proximal tubule epithelium,[23] in line with biochemical results suggesting that it is an important structural component of epithelia able to form a brush border. The presence of villin in only certain mammalian epithelia first indicated by immunofluorescence microscopy has been confirmed by the isolation of villin in milligram quantities from porcine enterocytes.[25] In addition, HT29, a permanent human cell line derived from colon adenocarcinoma, also expresses villin. Manipulation of the growth medium causes HT29 to further differentiate, which results in the appearance of a true brush border in the majority of the cells.[28]

In the current work a well-characterized antibody to villin[22] has been used in immunohistologic study for insight into the histogenesis of human renal cell carcinomas, because villin was found in proximal but not distal tubular epithelium in the mouse.[23] A second aim of the current study was to examine whether villin could help in the histologic grading of renal cell carcinomas. We also tested adenocarcinomas of the colon displaying different degrees of differentiation for the presence of villin to see whether villin could be used as a general differentiation marker for tumors that originate from villin-positive cells.

Materials and Methods

Tissues were snap-frozen in isopentane cooled to −140 C in liquid nitrogen as soon as possible after removal during surgery and stored at −70 C. For immunofluorescence assays cryostat sections nominally 5 μ in thickness were used. For staining with villin antibodies the sections were placed immediately into fixative F[23]: 3.7% formaldehyde, 2 mM ethylene glycol-bis-(2-aminoethyl ether)-N,N-tetraacetic acid, in phosphate-buffered saline for 3–4 minutes at room temperature. This fixative prevents the destruction of the microvilli which otherwise occurs in the presence of calcium.[24] These preparations were further fixed for 10 minutes in acetone at −10 C. For staining with keratin and vimentin antibodies, only the acetone step was used.

Antibodies

The villin antibody was raised against villin purified from chicken intestine.[22] It was affinity-purified on villin isolated from the same source and has been fully characterized elsewhere.[22]

The fimbrin antibody was raised against fimbrin purified from chicken intestine.[27] It was affinity-purified on fimbrin isolated from the same source and has been characterized elsewhere.[27]

The keratin antibody was a broad-specificity mouse monoclonal antibody—lu-5(von Overbeck et al., 1985). It was obtained from Dr. C. Stähli, Research Division, F. Hoffman-La Roche, Basel, Switzerland.

Two vimentin antibodies were available: 1) a mouse monoclonal, V-9[30]; 2) a guinea pig vimentin antibody.[33] Both have been characterized in detail elsewhere.

Isolation of a Crude Fraction Enriched in Villin From Human Material

A well-differentiated colon adenocarcinoma was selected and a cut assayed for villin by immunofluorescence microscopy. A small piece of tumor (<1 g) was extracted as follows so that we could obtain a supernatant fraction enriched in villin.[22] The tumor tissue was cut into small pieces and homogenized in 10 times its volume of Buffer I (75 mM KCl, 0.1 mM $MgCl_2$, 1 mM EDTA, 10 mM imidazole, pH 7.3) and further stirred in this buffer for 30 minutes at 4 C. After spinning for 10 minutes in an Eppendorf centrifuge, the pellet was extracted in five times its volume of Buffer II (75 mM KCl, 0.1 mM $MgCl_2$, 7.5 mM $CaCl_2$, 10 mM imidazole, pH 7.3) and again stirred for 30 minutes at 4 C. After again pelleting in the Eppendorf centrifuge, the supernantant was taken and a small aliquot was run on a 7.5% sodium dodecyl sulfate polyacrylamide gel. Proteins were transferred electrophoretically to nitrocellulose paper[31] and then tested with villin antibody that had been affinity-purified on villin isolated from chicken intestine (approximate concentration, 50 μg/ml), followed by gold-labeled anti-mouse IgGs. Amplification of the staining reaction was obtained by the use of the silver technique described by Moeremans et al.[32]

Results

Normal Human Colon and Adenocarcinomas of the Colon

In the normal human colon the affinity-purified antibody to villin revealed a sharply demarcated apical positivity in the brush border region of the lining epithelium (Figure 1b). Fimbrin showed a similar distribution in the epithelial cells and also stained other cell types. The columnar epithelium of the mucosa of the normal colon was strongly stained by broad specificity keratin antibodies (Figure 1a) but not by the vimentin antibodies.[33] Conversely the underlying mesenchymal cells were unstained by the keratin antibodies but strongly positive with the vimentin antibodies.

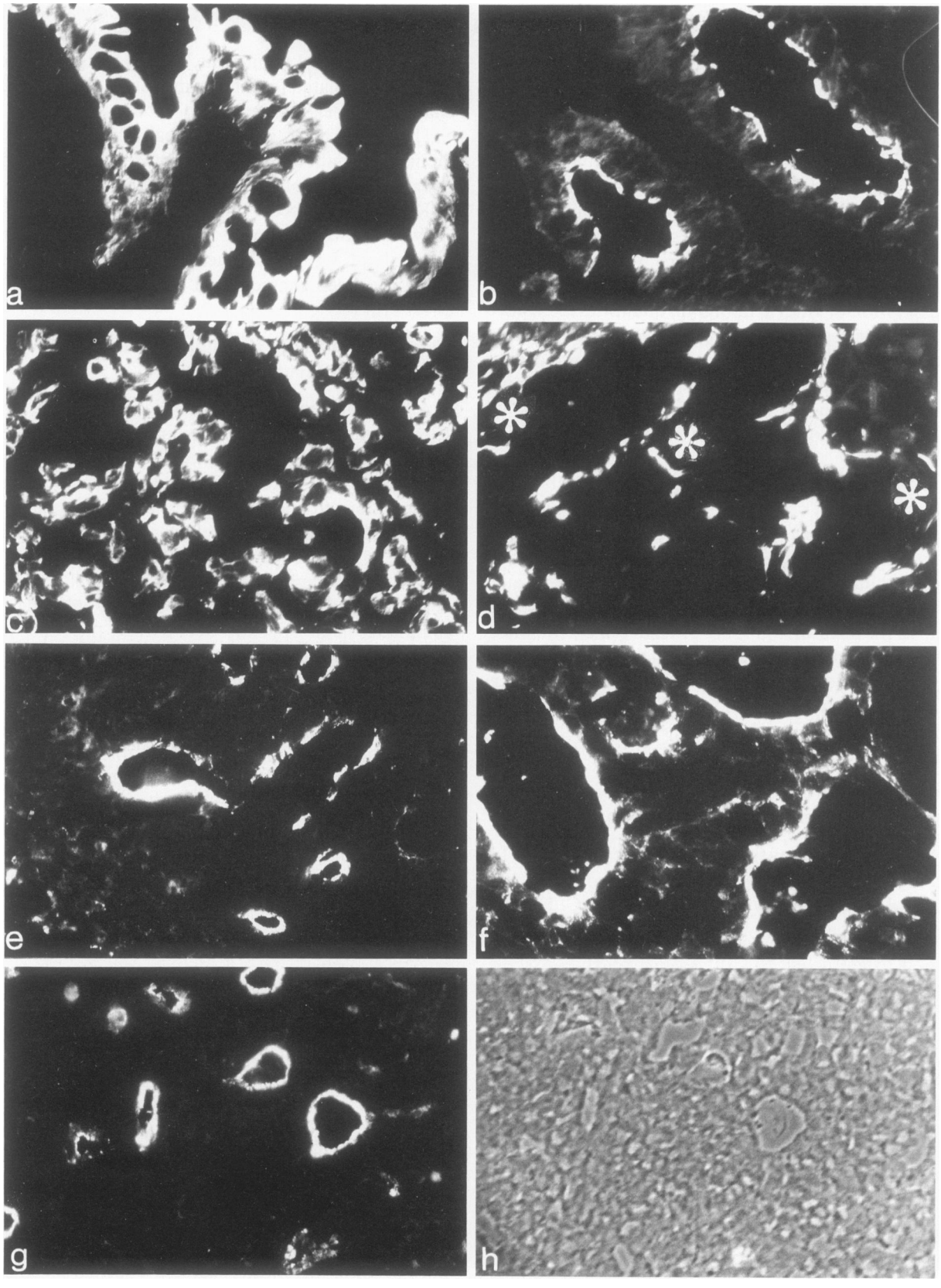

Figure 1—Immunofluorescence microscopy of human normal colon and adenocarcinoma of colon. **a**—Keratin antibody reveals the epithelial cells lining the surface and crypts of the normal colon mucosa. **b**—Villin antibody shows a distinct positive brush border staining of the regular colon mucosa. **c–f**—Adenocarcinoma of colon Grade II. **c**—Tumor cells are keratin-positive. **d**—Only the surrounding stromal cells, but not the tumor cells (*), are vimentin-positive. **e** and **f**—The apical portions of tumor cells of irregularly sized glandular structures stained strongly with fimbrin (**e**) and villin (**f**). **g** and **h**—Adenocarcinoma with poorly differentiated part. **g**—Only tumor cells forming adenoid structures demonstrated villin positivity; solid tumor nests were negative for villin. **h**—Corresponding phase-contrast micrograph. (**a** ×400; **b–h**, ×250)

Villin and fimbrin could be demonstrated in all tubular and glandular structures of the four adenocarcinomas of the colon that were examined (Figures 1e, f, and g, and Table 1). In each specimen the luminal part of the tumor cells showed a distinct positive rim. Undifferentiated parts of one colon adenocarcinoma without adenoid arrangement lacked villin positivity (Figures 1g and h). In these areas the tumor infiltrated the bowel wall in solid tumor sheets, gland formation did not occur; the tumor cells showed a pronounced nuclear polymorphism. It should be noted, however, that of four adenocarcinomas only one had regions of histologic Grade III. Further, poorly differentiated colon adenocarcinomas have to be studied so that we can see whether in such tumors villin expression is lost or reduced to a level too low to be observed by our immunofluorescence microscopy techniques. Adenocarcinomas of the colon were also tested with antibodies specific for keratin or vimentin. As expected from previous studies,[31,33] the tumor cells were keratin-positive and vimentin-negative irrespective of the histologic grade of the tumor.

Table 1—Distribution of the Actin-Associated Protein Villin and the Intermediate Filament Proteins Keratin and Vimentin in Adenocarcinomas of the Colon and in Renal Cell Carcinomas

Histologic grade	Number of Cases	Tumor cells positive for Villin	Keratin	Vimentin
Adenocarcinomas of the colon				
II*	4	4/4†	4/4	0/4
Renal cell carcinomas				
I‡	9	9/9	9/9	8/9
II	5	5/5	5/5	3/5
III	2	0/2	2/2	2/2

* Histologic grading[49]: I, well differentiated; II, moderately well differentiated; III, undifferentiated. One specimen was graded as II, focally III. The glandular part was villin-positive and the tumor sheets villin-negative.

† Left number indicates positive cases; right number shows total cases in one grade.

‡ For the grading system used for renal carcinomas see Table 2

Normal Human Kidney and Renal Cell Carcinomas

In sections of normal human kidney the brush border of the proximal tubular epithelium was strongly positive when tested with the villin antibody; in contrast, distal tubules, medullary tubular parts, and the glomeruli were negative for villin (Figures 2a and b). Fimbrin was also concentrated in the brush border of the proximal tubular epithelium and was negative on the distal tubular epithelium. In accord with previous results,[34-36] the broadly specific keratin antibody bound to tubular epithelia of proximal, distal, and medullary tubular segments as well as to the collecting duct epithelia and to epithelial cells of Bowman's capsule in a crescentlike fashion; the vimentin antibodies reacted with peritubular, glomerular, and vascular cells, but not with tubular epithelium.

Different renal cell carcinomas were examined with antibodies to villin, keratin, and vimentin. The results are summarized in Tables 1 and 2. Fourteen renal cell tumors showed positive staining for villin (Figures 2g, i, and k–n). Both well and moderately well differentiated renal cell carcinomas of histologic Grades I and II were positive for villin. This positivity was independent of the overall tumor structure; ie, villin positivity was found in papillary, tubular, and solid tumors. No correlation could be established between villin antibody reactivity and tumor cell types, in that tumors with both clear or granular cell types showed villin positivity. The grading system for antibody reactivity used in Table 2 indicates the percentage of tumor cells estimated to be positive for villin. The strongest villin positivity, reminiscent of that seen in normal proximal tubular epithelium, was seen in one renal carcinoma with a partly tubular, partly papillary growth pattern and with granular cells (no. 11, Table 2, Figure 2g). Here 75–100% of the tumor cells were villin-positive. In other instances when small glandular structures were present, a fine ringlike staining of the polar parts of the tumor cells was noted (eg, Figure 2i) and the percentage of tumor cells positive for villin was reduced. In other tumors, villin immunofluorescence was mostly focally accentuated and restricted to individual cells (eg, Figures 2i, and k–n).

Of the specimens in Table 2 where villin positivity was not detected two tumors were of the sarcomatous type (Figure 3c). Three additional renal cell carcinomas graded as well to moderately well differentiated carcinomas were villin-negative; the frozen sections stemmed from partly necrotic tumor tissue; one of these tumors had been embolized 24 hours preoperatively. When tested in immunofluorescence, these tumors were negative not only with villin but also only weakly positive or negative with other immunohistochemical markers, ie, keratin, vimentin, and fimbrin. These tumors are therefore not listed in Table 2.

Renal cell carcinomas that were villin-positive also had a polar cellular distribution for fimbrin (eg, Figure 2h); in addition, some stromal cells also were fimbrin-positive, in line with previous results showing that fimbrin is not restricted to the microvillus but is also found in other cell types.

In the majority of renal cell carcinomas (80%) tumor cells coexpressed keratin and vimentin (Figures 2e and f). In 20% of the renal cell carcinomas the tumor cells were keratin-positive and vimentin-negative: in

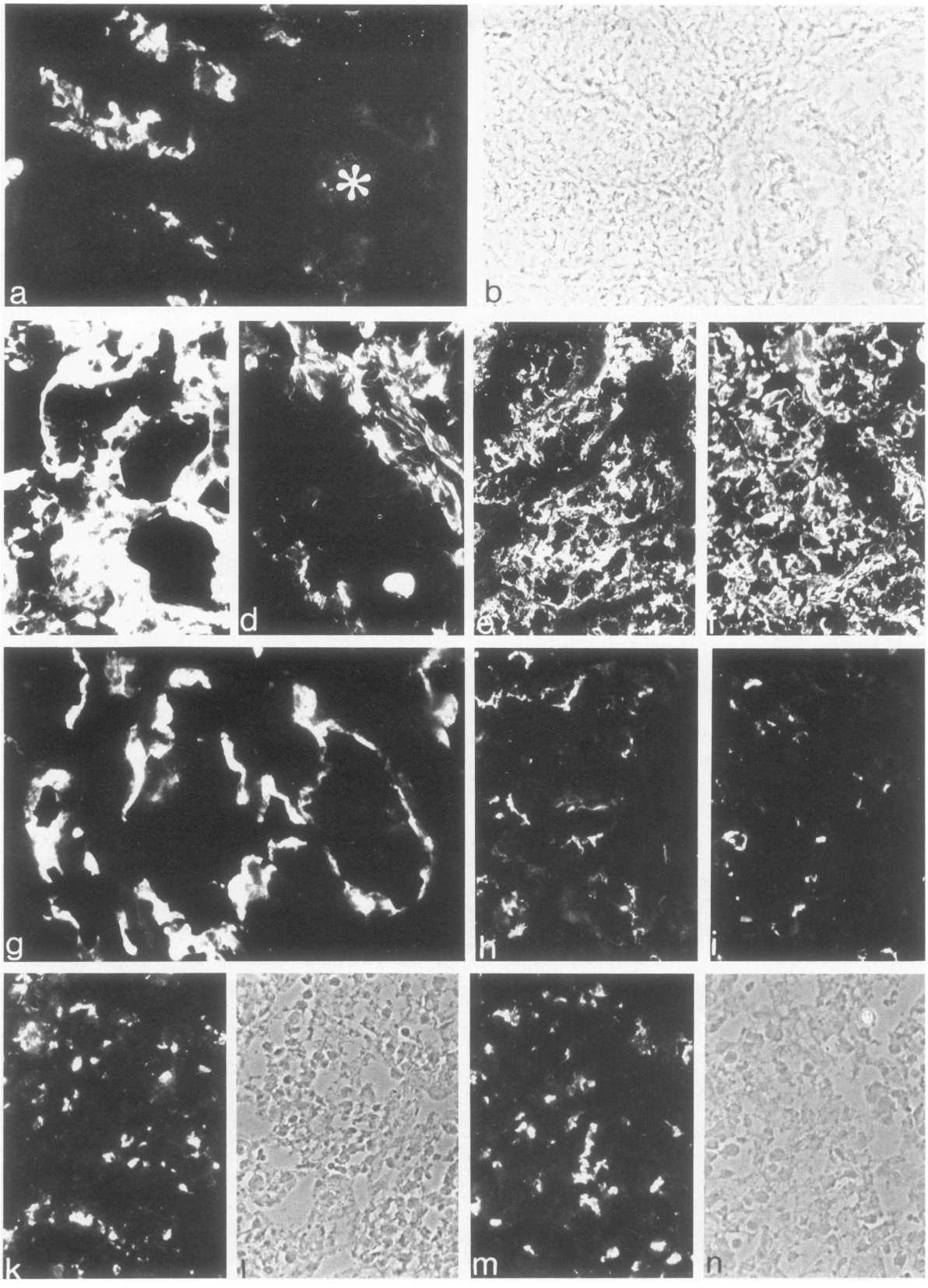

Figure 2—Immunofluorescence microscopy of human normal kidney and renal cell carcinomas. **a** and **b**—Villin antibody only stained the brush border region of proximal tubular epithelium. Glomerular cells and Bowman's capsule (*) are negative (**a**, immunofluorescence, **b**, phase-contrast). **c–n**—Renal cell carcinomas. **c** and **d**—Positive staining of clear and granular tumor cells with keratin only in **c**; tumor cells are negative for vimentin (**d**). **e** and **f**—Clear tumor cells react with antibodies to keratin (**e**) and with vimentin (**f**) antibodies. **g**—Villin antibody used on the same specimen as in **c** and **d** shows a strong irregular immunofluorescence of most tumor cells. **h** and **i**—Some tumor cell groups reveal a positivity, which is concentrated in microvilli, for fimbrin (**h**) and villin (**i**) of carcinoma of **e** and **f**. **k–n**—Immunofluorescence for villin in two other renal cell tumors (**k**, well differentiated; **m**, moderately differentiated) with corresponding phase-contrast micrographs (**l** and **n**). (×250)

Table 2—Immunocytochemistry of Renal Cell Carcinomas Using Antibodies to the Actin-Associated Protein Villin and to Keratin and Vimentin

Number* (age/sex)	Staging†	Histologic grade‡	Prevalent structure and cell type	Tumor cells positive for§		
				Keratin	Vimentin	Villin
1 (39 /M)	pT3	I	Trabecular; clear cells	++++	+	+
2 (47 /M)	pT2	I	Tubular; clear cells	++++	+→++	+
3 (54 /F)	pT2	I	Trabecular-tubular; clear cells	++++	++++	+
4 (55 /F)	pT2	I	Tubular-trabecular; clear cells	++++	–	+
5 (59 /M)	pT2	I	Trabecular-tubular; clear cells	++++	++++	(+)
6 (64 /F)	pT3	I	Trabecular; clear cells	++++	++++	+
7 (73 /F)	pT2	I	Tubular-trabecular; clear cells	++++	++++	+→++
8 (75 /M)	pT2	I	Trabecular; clear cells	++++	++++	+
9 (75 /F)	pT2	I	Trabecular; clear cells	++++	++++	++
10 (55 /F)	pT2	II	Trabecular-tubular; clear cells	++	++	++
11 (64 /M)	pT2	II	Tubular, papillary; clear-granular cells	++++	–	+++→++++
12 (66 /F)	pT2	II	Trabecular-tubular; clear cells	++++	+	++
13 (76 /M)	pT3	II	Papillary; granular-clear cells	++	++++	+
14 (80 /F)	pT3	II	Tubular; granular-clear cells	+++	ND	++
15 (64 /F)	pT3	III	Sarcomatoid	++++	++++	–
16 (59 /F)	pT4	III	Sarcomatoid	++++	++	–

* Tissue from three renal cell carcinomas did not show a reactivity to villin antibody. These tumors had either experienced a long time of warm ischemia or tissue sections stemmed from partly necrotic areas. Reactivity to intermediate filament antibodies also was weak or negative.

† The categories for staging are as follows[50]: pT1, a small tumor surrounded by renal parenchyma; pT2, a large tumor with deformity or calyceal or pelvic involvement and preserved continuity of cortex; pT3, spread of tumor into perinephric or peripelvic fat or hilar renal vessels.

‡ A histologic grading system modified from the one proposed by Griffiths and Thackray.[51] It is based on cytoplasmic and nuclear structure, degree of nuclear polychromasia, frequency of mitoses, and to a lesser degree the extent of papillary or adenomatous formations. Grade I is a well, Grade II a moderately, and Grade III a poorly differentiated carcinoma.

§ Immunofluorescence staining is assessed semiquantitatively: ++++, all cells are positive; +++, about 75%; ++, about 50%; +, less than 25% of cells are positive; (+), weak staining.

these specimens only the surrounding stromal cells were vimentin-positive (Figures 2c and d).

The data on different antibodies are summarized in Table 1. All 9 of the Grade I renal cell carcinomas contained villin-positive cells, although the percentage of tumor cells positive for villin in these tumors was usually less than 25%, and only in two instances in the 25–50% range (Table 2). In these tumors the tumor cells were keratin-positive, and in 8 of the 9 tumors also vimentin-positive. All 5 of the Grade II renal carcinomas

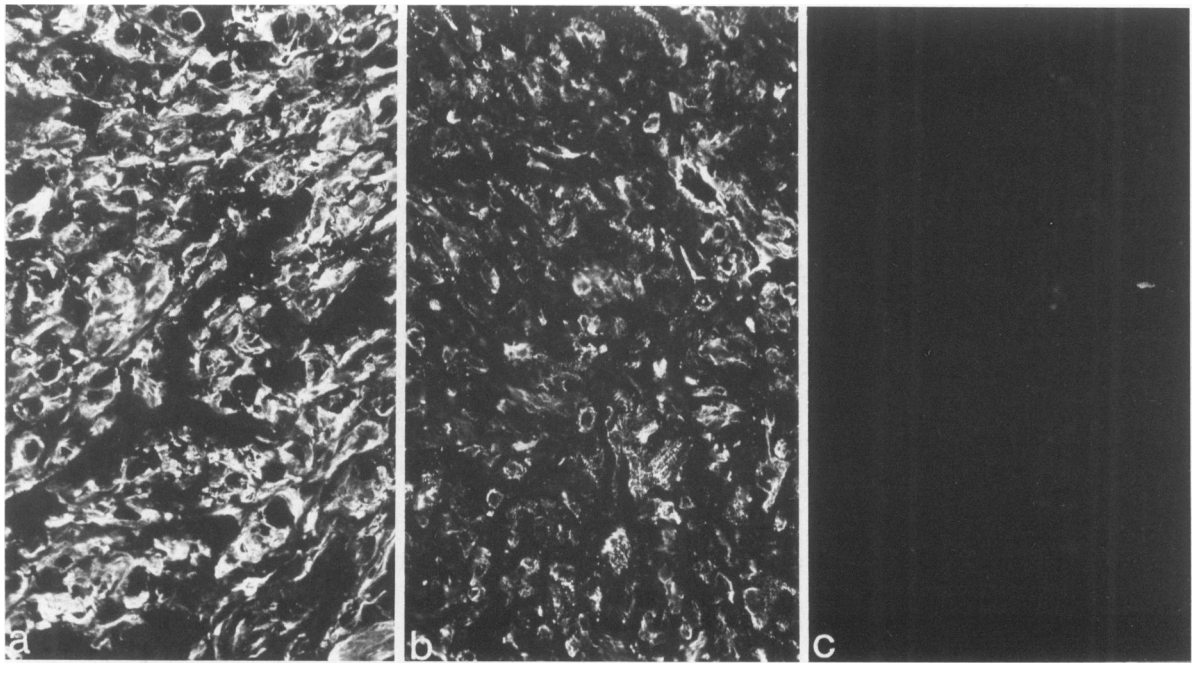

Figure 3—An undifferentiated renal cell carcinoma (**a–c**). Note that tumor cells are positive for keratin (**a**) and vimentin (**b**), but that the tumor cells do not show villin staining (**c**). (×250)

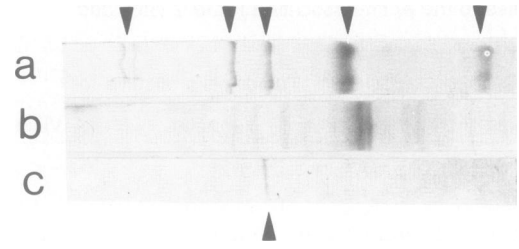

Figure 4—Immunoblotting of villin in an extract from human colon adenocarcinoma. **a**—Standards—from left spectrin doublet, galactosidase, phosphorylase b, bovine serum albumin, ovalbumin. **b**—Coomassie blue-stained gel of the polypeptides from the calcium supernatant separated by SDS gel electrophoresis. **c**—Immunoblot of a parallel slot to **b** using an affinity-purified villin antibody. Note that this antibody recognized a single band in the extract with a molecular weight of approximately 92 kd.

also contained villin-positive cells. One tumor had 75–100% villin-positive tumor cells, 3 tumors about 50% villin-positive tumor cells, and only 1 of the tumors had less than 25% villin-positive tumor cells. The tumor cells were again keratin-positive and in 3 out of 4 instances also vimentin-positive. Interestingly, the tumor with the 75–100% villin-positive tumor cells had vimentin-negative tumor cells. Tumor cells of the two Grade III renal carcinomas were negative for villin but positive for keratin and vimentin (Figures 3a–c).

Immunoblots on Human Villin

The villin antibody used in this study was originally raised against villin purified from chicken intestine. The immunofluorescence results reported above suggest strongly that the antibodies recognize human villin, because the distribution in sections from human colon and kidney is similar to that reported previously with the same antibody on rat and chicken tissues.[22,23] To confirm this, immunoblotting was performed using the villin antibody and a partially purified supernatant fraction isolated from a moderately well differentiated adenocarcinoma of the colon (see Materials and Methods). The results are shown in Figure 4. The villin antibody identifies a single polypeptide of an approximate molecular weight of 92 kd in the calcium-containing supernatant (slot c). Thus, the villin antibody used here recognizes a human protein of appropriate molecular weight in a biochemical assay.

Discussion

In the present study renal cell carcinomas were investigated using a specific antibody to villin, a protein that only occurs in epithelial cells with well-developed microvilli. Villin is the best characterized of the brush border-specific proteins, and its mechanism of action at the molecular level is understood. Villin could be demonstrated by immunohistology in 14 renal cell carcinomas (Tables 1 and 2), thus showing that some tumor cells share an antigen found only on the proximal tubular cells of the normal adult kidney. In 3 of the 5 villin-negative tumors examined, the lack of villin staining seems to have been caused by ischemia. Electron microscopic examination of experimental renal ischemia[37,38] as well as of human material[39] which has undergone ischemia shows irregularity and distortion of microvilli. In these three instances necrotic areas in parallel conventionally stained sections of the tumor specimens were present. Intracellular calcium concentrations may increase during ischemia,[40] and at high calcium concentrations villin cleaves F-actin, thus destroying the core microfilament bundle and therefore the microvillus structure.[24] Thus, ischemia is probably a sufficient explanation for the lack of positive staining by villin antibodies in these three specimens. In the two undifferentiated renal carcinomas where well-preserved tissue was available, villin immunofluorescence was negative.

How does the degree of differentiation as assayed by the percentage of villin-positive tumor cells correlate with the histologic grading systems currently in use for renal cell carcinomas? Although villin positivity seems to separate renal cell carcinomas placed in Grades I and II by the grading system used in this study from the sarcomatous Type III, there is little correlation of villin positivity when different tumors graded as Types I and II are compared. Villin staining was seen independent of the appearance of the tumor cell cytoplasm or nucleus. It was evident in our study that villin reactivity of renal cell tumors occurred primarily in some, but not all, tumor cells. In a few instances tubular structures showed a ringlike positivity (see Figure 2i). This scattered reactivity pattern on renal carcinomas contrasts with the strong villin staining in apical portions of tumor cells in the glandular structures of moderately differentiated adenocarcinomas of the colon. In undifferentiated parts of colon adenocarcinomas villin could not be detected (Figure 3c), and this in colon is again consistent with the idea that villin expression is characteristic of the more differentiated tumor cells. Robine et al,[41] in a study appearing after this manuscript was submitted for publication, have shown by biochemical methods that adenocarcinomas of the intestine are villin-positive. Eleven of the 12 human adenocarcinomas studied were villin-positive.

Renal cell tumors composed mainly of granular cells have been thought to have a worse prognosis than clear cell carcinomas.[17] Interestingly, the only tumor in which villin expression was seen in almost all tumor cells was a renal cell carcinoma composed of granular as well

as clear cells; also, other tumors with granular cells showed strong villin positivity, whereas some clear cell carcinomas revealed villin only in a few cells. Therefore, perhaps granular cell carcinomas are not necessarily more undifferentiated tumors than clear cell carcinomas. Other investigators came to the same conclusion by comparing ultrastructural features of granular and clear cell carcinomas.[15,42]

Our results with villin support studies[10,11,43-45] in which renal cell carcinomas have been shown to express antigens found on proximal but not on distal tubules of the normal adult kidney usually by immunologic techniques. Antigens used have included some present in crude and biochemically undefined brush border fractions,[11,44] some identified by monoclonal antibodies raised against cell surface antigens of cultured human renal cancer cells[45] and those recognized by certain lectins.[10] A similar search for antigens associated with distal tubules such as the Tamm-Horsfall antigen has usually proved unsuccessful.[10] How to interpret a recent study in which 28 of 30 renal carcinomas were positive for epithelial membrane antigen (EMA)[14] is not clear. Although EMA is associated with distal tubules in the normal adult kidney,[14,46] expression of EMA has been noted in proximal tubules of damaged kidneys.[46] It is also not clear how to interpret the finding that renal cell carcinomas very often coexpress keratin and vimentin, whereas in normal human kidney both the proximal as well as the distal tubules reveal only keratin. In our study 80% of the carcinomas showed such coexpression, whereas in previous studies this number has been reported as either 57%[10] or 100%.[48] In addition, a heterogeneity in the percentage of tumor cells expressing different markers in different tumors has been noted not only for villin but also for other antigens. In summary, the data suggest that renal carcinoma cells may be arrested at a stage of cellular differentiation in which certain brush border antigens typical of proximal tubules can be expressed in some cells but where expression of Tamm-Horsfall antigen is not possible. Whether renal cell carcinomas expressing villin or other brush border antigens originate from transformation of fully differentiated proximal tubule cells (which might then dedifferentiate and acquire vimentin—a similar proposal for mesothelial cells was made by Connell and Rheinwald[47]) or from an as yet undescribed precursor cell (which could coexpress keratin and vimentin) remains to be determined. We noted that a certain fraction of renal carcinomas, in particular, the sarcomatous renal cell carcinomas, were negative for villin (compare other studies where particular antigens have been found to be lacking in a minority of renal carcinomas[10,43]). The histogenetic origin of these villin-negative tumors remains an open question.

In conclusion, our results support the idea that villin could serve as a marker of tumor cell differentiation with villin-positive tumors being more differentiated than villin-negative ones. Whether one can further grade tumors by determining the percentage of tumor cells that are villin-positive remains to be determined. In principle, well-defined immunohistologic differentiation markers such as villin could help to achieve a more objective tumor grading system. However, future studies have to show whether a histologic grading system that combines established grading criteria with the existence and intensity of villin staining is also useful as a prognostic aid.

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Acknowledgments

We thank Susanne Isenberg for expert technical assistance.