Virchows Archiv A Pathol Anat (1988) 413:303–312

Virchows Archiv A
Pathological Anatomy and Histopathology

Villin, intestinal brush border hydrolases and keratin polypeptides in intestinal metaplasia and gastric cancer; an immunohistologic study emphasizing the different degrees of intestinal and gastric differentiation in signet ring cell carcinomas

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Summary. Gastric carcinomas have been assayed for the presence of villin and for the small intestinal hydrolases aminopeptidase N and sucrase isomaltase. These proteins seem not to be present in normal stomach epithelium. However intestinal metaplasia in stomach, and tumour cells in the glandular patterns of gastric carcinoma were positive for all three markers, showing characteristic apical positivity. In contrast, in diffuse gastric carcinomas the percentage of signet ring cells positive for these markers varied from 10–100% with each marker showing a similar percentage of positive cells. Testing of gastric carcinomas with antibodies specific for different keratin polypeptides showed that while all 7 tumours were positive for keratins 8 and 18,2 were also positive for keratin 7. In the keratin 7 positive tumours all tumour cells were keratin 7 positive. The keratin 8 antibody also reacted on routinely fixed specimens. Thus gastric carcinomas reveal different degrees of gastric and intestinal differentiation

Key words: Signet ring cell carcinoma – Villin – Small intestinal hydrolases – Gastric carcinoma – Keratins

Introduction

Human gastric carcinomas can be classified into intestinal and diffuse types (Lauren 1965). The intestinal type includes papillary adenocarcinomas and tubular adenocarcinomas and these display well-differentiated tubules or glands composed of large columnar cells. The diffuse type, includes signet ring cell carcinomas (SRC) and poorly differentiated adenocarcinomas. Here the tumour cells are scattered either singly, or in small clusters, or are aggregated into primitive glandular structures. The intestinal type is thought to arise from metaplastic gastric epithelium (Morson 1955; Lauren 1965; Nakamura 1968; Johansen 1981) while the diffuse type is believed to originate from the indifferent cell zone of non-metaplastic mucosa (Nagayo 1961, 1965, 1974, 1975; Nakamura 1968; Hattori 1985). Previous studies have shown intestinal properties in some signet ring cell carcinomas thus supporting a possible intestinal origin (Lauren 1967; Häkkinen et al. 1968; De Boer et al. 1969; Järvi et al. 1975; Santini et al. 1987). Intestinal metaplasia can be found associated with both intestinal and diffuse gastric carcinomas but is also commonly observed in many benign diseases of the stomach.

Recent biochemical and immunohistological studies have examined the phenotypic expression of certain cytoskeletal proteins in tumour cells in human gastrointestinal carcinomas. Thus intermediate filament typing has shown that both gastric and intestinal carcinomas express keratin. This expression is independent of the degree of differentiation (Altmannsberger et al. 1982; Moll et al. 1982; Osborn et al. 1985). Other markers are restricted to tissues that show brush border differentiation. Thus villin, a 95 Kd molecular weight cytoskeletal protein, first isolated in 1980 (Bretscher and Weber 1980), is one of the four major F-actin associated proteins, characterizing the core microfilament

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bundle of the microvillus. Villin is found in the intestinal epithelium and renal proximal epithelium (Bretscher et al. 1981; Robine et al. 1985). Tumour cells of the majority of intestinal and renal carcinomas also stain positively for villin (Robine et al. 1985; Gröne et al. 1986; Moll et al. 1987, Pitz et al., 1987). Other markers which locate to and positively identify brush border containing structures include aminopeptidase N (APN), and sucrase isomaltase (SI) (Hauri et al. 1985). These hydrolyases which in the adult are restricted to the small intestine can also be detected by immunological techniques in fetal colon and in 7/27 human colon carcinomas (Zweibaum et al. 1984).

In order to gain further insight into the complex phenotype expression and histogenesis of human gastric cancer we have examined gastric carcinomas fixed by different methods with antibodies specific for particular keratin polypeptides. We have also assayed gastric carcinomas and intestinal metaplasia for the presence of villin, aminopeptidase N and sucrase isomaltase to see whether they can be used as general markers of intestinal differentiation in benign and malignant gastric tissues.

Materials and methods

The sixteen specimens of advanced gastric cancer used in this study were classified histologically according to the criteria established by Lauren (1965) (Tables 1 and 2). In 7 instances biopsy material was obtained during surgery and immediately snap-frozen in isopentane cooled to −140° C in liquid nitrogen, with subsequent storage at −70° C. For immunofluorescence assays cryostat sections ~5 microns in thickness were used. A further 9 diffuse type carcinomas in which signet-ring cells were present were obtained from the files of the Department of Pathology, University of Bologna (Table 2). These were gastrectomy specimens that had been routinely fixed in formalin

Table 1. Reactions of frozen sections of gastric carcinomas with keratin antibodies and with brush border markers

Specimen no		1	2	3	4	5	6	7	NI[e]	NS[e]
Sex/age (year)		f/77	m/64	f/77	m/58	m/76	m/80	f/59		
Histological diagnosis										
GC		I	M	D[c]	D	D[a]	I[b]	I		
SRC[f]		np	+	+	+	+++	np[d]	np		
IM[f]		++	np	+++	np	+	np	np		
Keratin antibodies[g]										
KL1 (broad)		+	+	+	+	+	+	+	+	+
CK8 (8)		+	+	+	+	+	+	+	+	+
CK2 (18)		+	+	+	+	+	+	+	+	+
CK7 (7)		−	−	−	−	−	+	+	−	−
Brush border markers[g]										
Villin	SC	np	30–50%	10%	50–70%	50–100%	10%	np		
	glandular pattern	+	+	+	np	np	+	−	+	−
	IM	+	np	+	np	+	np	np		
APN	SC	np		10%	50–100%			np		
	glandular pattern	+		+	np		−	−	+	−
	IM	+		+	np			np		
SI	SC	np		10%	50–100%			np		
	glandular pattern	+		+	np			−	+	−
	IM	+		+	np			np		
AP	IM			+					+	−

Abbreviations. NI normal intestine, NS normal stomach, (corpus) GC gastric carcinoma, I intestinal type, D diffuse type, M mixed type, SRC signet ring carcinoma cells, IM intestinal metaplasia, SC single cells (no information as to fraction that are are SRC), APN amino-peptidase N, SI sucrose isomaltase, AP endogeneous alkaline phosphatase not inhibited by levamisole.

[a] gelatinous type
[b] poorly differentiated intestinal type microglandular variety
[c] some areas show mixed type morphology
[d] single cells (SC) present but did not show SRC morphology
[e] normal epithelia and glands.
[f] +++, ++, + indicates the frequency with which a particular structure was seen in the specimen
[g] + all tumour cells strongly and specifically stained, − structure present but not stained
np structure not present

Table 2. Reaction of formalin fixed paraffin embedded gastric carcinomas with keratin antibodies KL1 and CK8

Specimen no		11	12	13	14	15	16	17	18	19
Sex/age (yrs)		m/70	m/80	f/58	m/66	f/72	f/58	m/45	f/49	m/48
Months between fixation and staining		3	3	9	11	13	13	37	51	87
Histological diagnosis[a]	GC	D	D	D	D	D	D	D	D	D
	SRC	+	+++	+++	++	+++	+++	+	+++	+++
	IM	np	+	+	+	np	+	+	+	+
Keratin antibodies[b]										
KL1	IM	np	++	+/−	++	np	+/−	+/−	−	+/−
	SC	++	++	+/−	++	++	+	−	+/−	+
CK8	IM	np	+	+/−	++	np	+	+/−	+/−	+/−
	SC	++	+	+/−	++	++	+	−	+/−	+/−

For abbreviations see Table 1.
[a] +++, + indicates the frequency with which a particular structure was seen in the specimen
[b] +++ strong ++ medium + weak positivity all cells, +/− only some cells weakly positive − no reaction

and then paraffin embedded 3–87 months previously. Paraffin sections ~5 microns thick were cut, dried for 12–18 h at 37° C and then deparaffinized.

The fixation used depended on the antigen to be studied. For keratin antibodies both cryostat and paraffin sections were fixed with acetone at −10° C for 6 min (method 1). Preservation of the brush border antigens in frozen sections was additionally assayed by transferring the sections immediately after cutting and before reaching room temperature into a buffer containing 3.7% formaldehyde, 2 mM ethylene glycol bis (2 aminoethyl) N,N,N^1,N^1-tetraacetic acid in phosphate buffered saline (no Ca^{2+}, no Mg^{2+}) for 2 min at room temperature with subsequent fixation in acetone at −10° C for 6 min (method 2). Alternatively sections were transferred to 1% formaldehyde in phosphate buffered saline for 10 min at room temperature with subsequent fixation in acetone at −10° C for 6 min (method 3). Immunofluorescence microscopy was by standard procedures using commercial FITC labelled second antibodies (Cappell Laboratories, Cochraneville, Pa, USA).

Antibodies used were:
1) the broad specificity keratin antibody KL1 (Dianova, Hamburg, FRG) used as hybridoma supernatant diluted 1:10 (Viac et al. 1983); 2) the keratin 8 specific clone CK-8 (Osborn et al. 1986) used as a hybridoma supernatant. 3) the keratin 18 specific clone CK2 (Debus et al. 1983); 4) the keratin 7 specific clone CK7 (Tölle et al. 1986; Osborn et al. 1986); 5) a rabbit antibody against native chicken villin. This antibody was made monospecific for villin on Sepharose 4B and used at an approximate concentration of 50 µg/ml (Bretscher and Weber 1980; Glenney et al. 1982; Gröne et al. 1986). 6) aminopeptidase N antibody (HBB 3/153/63, ascites fluid diluted 1:300) (Hauri et al. 1985). 7) Sucrase isomaltase antibody (HBB 2/614 188, ascites fluid diluted 1:300) (Hauri et al. 1985).

To examine endogeneous alkaline phosphatase activity frozen sections were fixed by method 2. They were washed with Tris buffer and developed using napthol AS-MX/fast red as the substrate in the presence of levamisole which inhibits the majority of endogeneous alkaline phosphatase present in human tissues, but does not inhibit the intestinal alkaline phosphatase (Ponder and Wilkinson 1981). Development was by standard procedures (Dako, Klostrup, Denmark).

Results

Keratin antibodies

The results of staining frozen sections of gastric carcinomas with either broad specificity keratin antibodies (e.g. KL1) or with antibodies specific for individual keratin polypeptides are summarized in Table 1 and Fig. 1a–g. As noted previously, tumour cells in both intestinal and diffuse carcinomas are positive with broad specificity keratin antibodies such as KL1. This was true for intestinal type gastric carcinomas (Fig. 1a), for mixed and diffuse types (Fig. 1b) as well as for tumours in which signet ring carcinoma cells were present (Fig. 1d, e). Intestinal metaplasia was also positively stained (cases 1, 3, 5 Table 1). When antibodies specific for keratin 8 (CK8) or for keratin 18 (CK2) were used, all tumour cells were again positive, as was intestinal metaplasia. This is illustrated in Fig. 1f and in Fig. 2g for carcinomas stained with the CK2 antibody. Of the seven tumours tested with the keratin 7 specific antibody (CK7) in Table 1, two were positively stained while five were not stained by this antibody.

Relatively few keratin antibodies react after formaldehyde treatment of cells or tissues. However as shown in Table 2, KL1 and CK8 react with cells or tissues which had been fixed with formaldehyde, while CK2 and CK7 do not. KL1 and CK8 were therefore used on 9 gastric carcinomas that had been routinely fixed in formaldehyde and embedded in paraffin. Results are summarized in Table 2 and in Fig. 1 (h–j). Clear keratin positivity was demonstrated in intestinal metaplasia

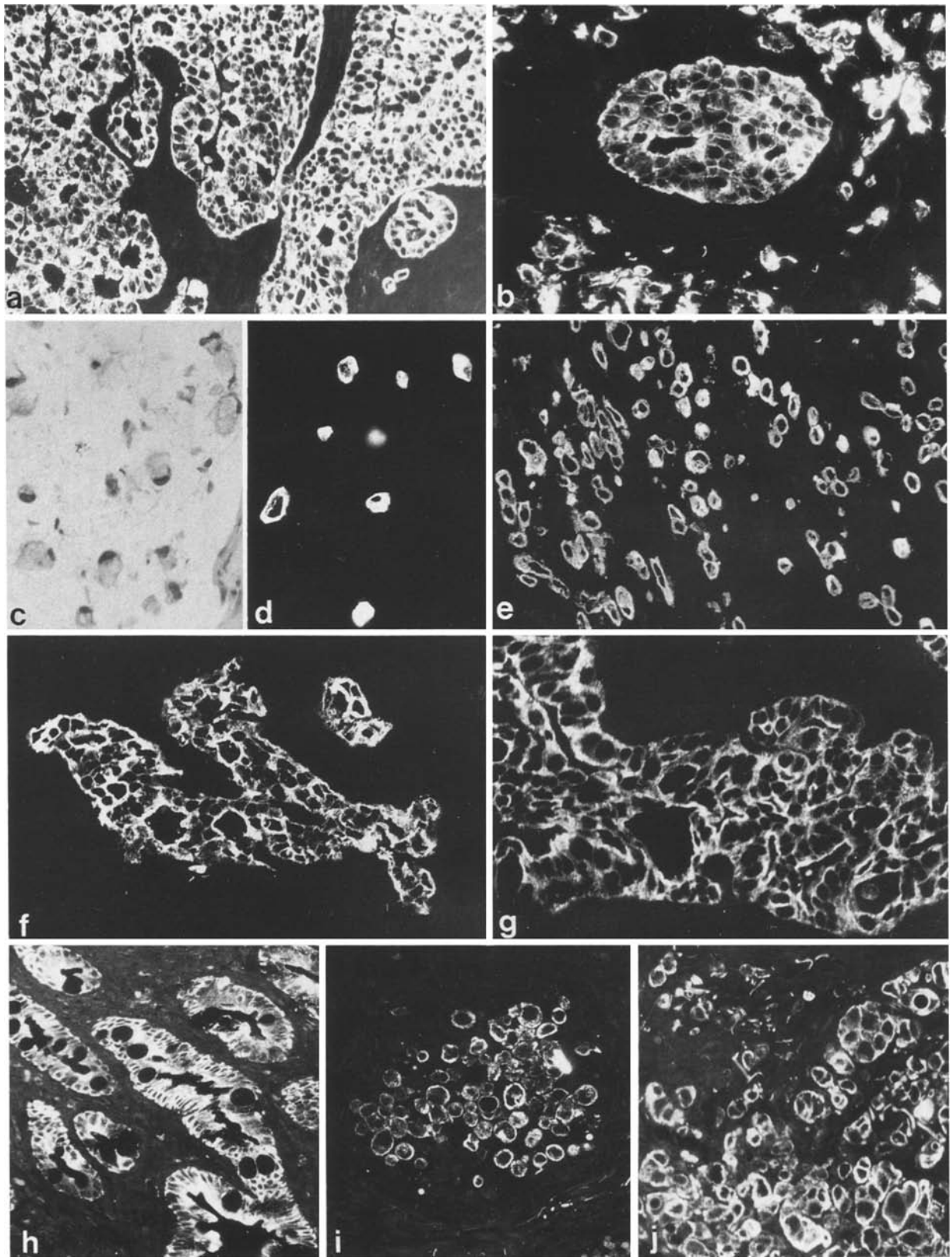

Fig. 1. Keratin positivity of gastric carcinomas (**a–g**) cyrostat sections. (**h–j**) formalin fixed paraffin embedded specimens. (**a, b**) case 3, (**d**) case 5 and (**e**) case 4 are stained with the broad specificity keratin antibody KL1. (**c**) case 5 haematoxylin eosin stain. (**f, g**) case 6 stained with (**f**) CK2 antibody specific for keratin 18 and (**g**) CK7 antibody specific for keratin 7. (**h, i, j**) case 14 stained with (**h, i**) broad specificity KL1 keratin antibody or (**j**) CK8 specific for keratin 8. Note that all tumour cells irrespective of whether they are found in glandular (**a**) microglandular (**f, g**) diffuse (**b**) or in which signet ring cells are visible (**b, d, e, i, j**) are keratin positive. Intestinal metaplasia (**h**) is also keratin positive (see Table 1). While tumour cells and intestinal metaplasia were always positive with KL1, CK2, and CK8 antibodies, only 2 of 7 tumours were positive with the CK7 antibody (**g**). Fixation (**a, b, d, e**) Method 2 (**f, g**) Method 1. (**h, i, j**) deparaffinization, acetone. Magnifications (**a, h, i, j**) ×150 (**b–g**) ×250

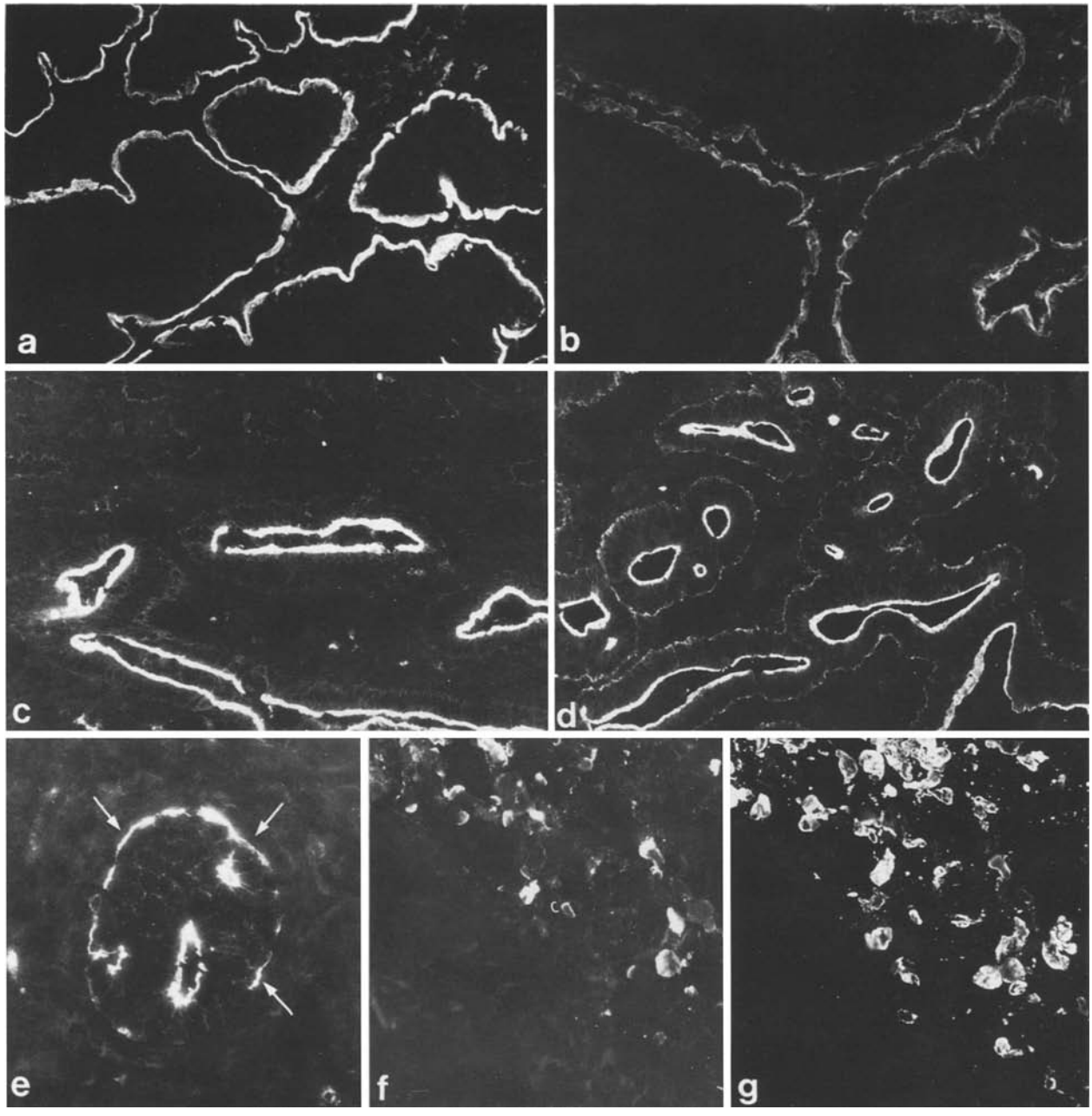

Fig. 2. Reaction with villin antibodies (**a, b**) of normal intestine. (**c**) Intestinal metaplasia (case 1) and (**d**) carcinomatous glandular pattern (case 1). Note positive staining of microvilli, and that villin is highly enriched in this part of the epithelial cells. (**a, b**) are fixed by different methods and have been then photographed and reproduced using similar exposures so they are directly comparable. (**a**) fixation method 2 (**b**) method 1. Villin antibodies react much more strongly with (**a**) than (**b**) where most of the villin has been lost (see text). (e) Villin stain of tumour cells that have invaded a lymph vessel in case 3. Note the concentration of villin not only in the brush border bordering the lumen, but also (*arrows*) at the border in contact with the lymph vessel walls. (**f, g**) Double immunofluorescence microscopy of infiltrating single tumour cells from case 2 with (**f**) villin and (**g**) keratin CK8 antibody. Note that some cells are positive for both villin and keratin. Fixation method 2. Magnifications (**a–d**) ×150, (**e–g**) ×250

(Fig. 1h) as well as in signet ring cell carcinoma cells (Fig. 1i, j). As seen in Table 2 the reaction on both intestinal metaplasia and on single tumour cells, was specimen dependent. More recently fixed specimens showed a stronger reaction than older specimens, but in general the staining of all specimens was somewhat weaker than in the frozen specimens.

Brush border markers

1. Villin. Preservation of microvilli, and in consequence of the brush border, is critically dependent on fixation. It has been documented for both animal and human tissues, that to get good preservation of microvilli the original specimen or the sections must be fixed immediately in formaldehyde or glutaraldehyde. This is because villin severs the F-actin filaments of the microvilli leading to their destruction at calcium concentrations $>10^{-6}$ M (Bretscher and Weber 1980). The difference between fixation with formaldehyde and EGTA, and acetone fixation is documented in Figs. 2a and b, which were subsequently stained with antibodies to villin and photographed under identical conditions. The staining is very much stronger in the sample fixed with formaldehyde and EGTA than in the sample fixed in acetone (although if Fig. 2b is further exposed some villin staining will be seen). This point is documented, because of a recent claim that the full complement of villin is preserved in cells and tissues fixed only by acetone (Moll et al. 1987).

Villin positivity is characteristic of intestinal but not stomach epithelium (Bretscher et al. 1981; Robine et al. 1985 and Table 1). In gastric carcinoma, intestinal metaplasia is always strongly positive with the staining restricted to the microvillus region (Fig. 2c). In gastric carcinoma of the intestinal type, villin positivity is present where the tumour cells border the lumen (Fig. 2d). In one instance tumour cells had invaded a lymph vessel. Here, interestingly, villin positivity was noted not only on the apical side of the tumour cells bordering the lumen but also on the side of the tumour cells abutting on to the walls of the lymph vessel (Fig. 2e). When diffuse carcinomas with signet ring cells were examined a variable percentage of the signet ring cells stained positively with keratin antibody KL1 or CK8, or by double label immunofluorescence microscopy using villin and keratin antibodies on the same section (Fig. 2f, g). The percentage of tumour cells that were villin positive varied from 10% in specimens 3 and 6 to 50–100% in specimen 5.

No reaction was obtained with the villin antibodies on two routinely fixed and paraffin embedded gastric carcinomas, which had stained strongly with keratin antibodies (data not shown).

2. Small intestinal hydrolases. Frozen sections were assayed with antibodies to aminopeptidase N and sucrase isomaltase by the same three fixation methods used for villin. The results are shown in Table 3. Method 2 was adopted since it gave the sharpest staining of the microvilli region and allowed the results to be compared directly with those obtained with villin antibodies. Results are presented in Table 1 and in Figure 3. The microvilli of normal intestine were strongly stained with both antibodies (Fig. 3a, b) (cf. Hauri et al. 1985), whereas normal stomach epithelia were not stained (Table 1). Intestinal metaplasia was always strongly positive (Fig. 3c, Table 1). When diffuse carcinomas were examined, a variable percentage of the single tumour cells were positively stained. An example in which nearly all cells appeared positive with the APN antibody is shown in Fig. 3d. The percentage of single cells that were positive with both antibodies varied from 10% in specimens 3 and 6 to 50–100% in specimen 4.

Table 3. Effect of fixation conditions on villin, aminopeptidase N and sucrase isomaltase reactivity

	Method 1	Method 2	Method 3
Villin	+D	+++S	+D
Aminopeptidase N	++W	+++S	++D
Sucrose isomaltase	+D	+++S	+D

Method 1 acetone; Method 2 3.7% formaldehyde, 2 mM EGTA in PBS, acetone; Method 3 1% formaldehyde in PBS, acetone
For further details see text.
+++ very strong staining, S sharp border (cf. Fig. 2a) and + weaker staining D more diffuse (cf. Fig. 2b) ++W in between with a wider microvilli region

No reaction was obtained with either antibody on the same formaldehyde fixed, paraffin embedded gastric carcinomas tested with the villin antibody.

3. Endogenous alkaline phosphatase. Levamisole does not inhibit the intestinal alkaline phosphatase, although it does inhibit alkaline phosphatases in other tissues (Ponder and Wilkinson 1981). We therefore wondered whether this procedure could yield further information as to a gastric or an intestinal differentiation. Under our conditions phosphatase reaction was found in the intestine where it was restricted to the microvilli of the epithelium, but was not found in normal stomach epithelium. Intestinal metaplasia (case 3) was strongly positive.

Discussion

Intestinal versus gastric differentiation

Intestinal metaplasia is characterized by the presence of villin, APN, and SI, and endogeneous AP

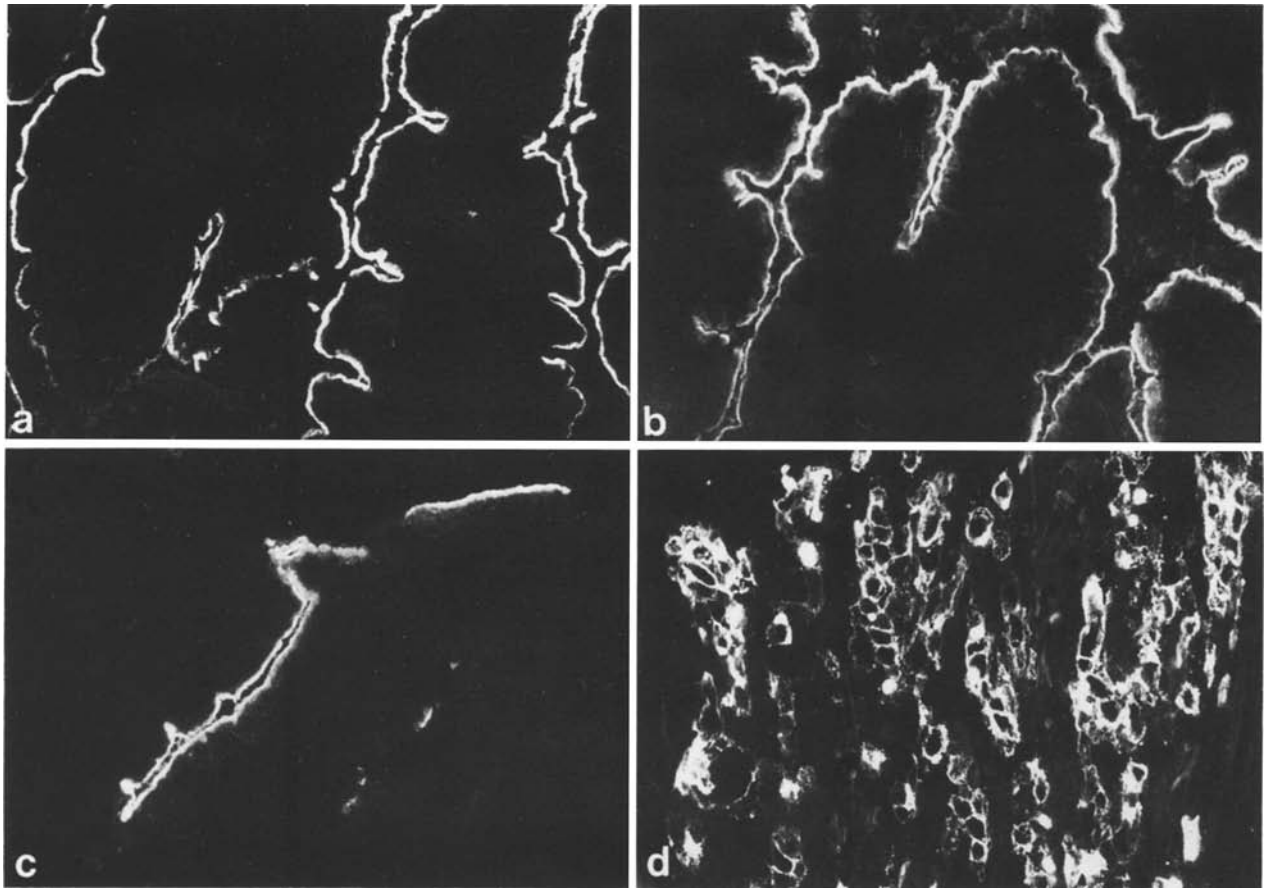

Fig. 3. Reaction with antibodies to sucrase isomaltase (**a, c**) or to aminopeptidase N (**b, d**) of either normal intestine (**a, b**), intestinal metaplasia (case 3), and of single infiltrating tumour cells (case 4) (**d**). In (**a–c**) the positive reaction is confined to the region of the microvilli. Fixation method 2. Magnification (**a–i**) ×150, d ×250

as are the tumour cells in the glandular pattern of gastric carcinomas. In both instances villin and the intestinal hydrolases are found in the microvilli region which borders the lumen. In contrast, when gastric carcinomas of the diffuse type are examined, individual tumour cells can, but need not, express the brush border markers. Thus in the different tumours listed in Table 1 a variable percentage of tumour cells express villin, APN or SI. Interestingly, however, for a given tumour the percentage of cells positive for each of these markers appears approximately similar. This percentage ranges from 10% (case 3 and 6) to 50–100% (case 4). Positive single cells no longer show a polarized distribution of these markers.

Moll et al. (1987) have also recently noted villin expression in tubular adenocarcinomas of the stomach. As in our specimens, villin positivity was predominantly apical. Villin positivity of single signet ring cells was not noted by these authors, although we found such staining in a varying percentage of tumour cells in all our signet ring cell carcinomas. This difference could perhaps be due to the different fixation procedures used (c.f. Fig. 2a, b). Stomach epithelium has been reported as negative for villin in rat (Robine et al. 1985). Moll et al. (1987) noted that the corpus is negative but report weak positivity of the antrum for villin in man. This is a little surprising in view of the ultrastructural findings that the surface epithelium in the antrum mucrosa does not differ from the surface epithelium of the mucosa in the gastric body (Nevalainen and Jarvi 1977) both being characterized by short stubby microvilli lacking central cores in contrast to the longer and denser microvilli typical of normal intestine and intestinal metaplasia. However the possible villin positivity of the antrum microvilli is interesting in that intestinal metaplasia is often preferentially located in this region of the stomach (Lei and Yu 1984). Adenocarcinomas of many other sites are villin negative (Robine et al. 1985; Louvard et al. 1987).

There seems to be an inverse relation between the expression of the three intestinal markers and the expression of keratin 7. Thus none of the cases where 30–100% of the tumour cells were villin, APN, or SI positive were keratin 7 positive. In contrast in case 6 where only 10% of the tumour cells showed the intestinal markers all tumour cells expressed keratin 7. We note that in our series, two tumours showed keratin 7 expression. Although keratin 7 expression has been noted neither in normal stomach (Osborn et al. 1986; Ramaekers et al. 1987) nor in three adenocarcinomas of the stomach assayed with keratin 7 specific antibodies (Osborn et al. 1986), one of two stomach adenocarcinomas assayed by 2D gels contained a small amount of keratin 7 (Moll and Franke 1986). Whether keratin 7 expression can be used to identify a "subtype" of gastric carcinoma should be further studied with a larger series of specimens.

The above criteria can be added to others well established in the literature. Intestinal metaplasia can be incomplete if only absorptive cells are present, or complete if in addition goblet cells containing mucus granules and Paneth cells are present. At the ultrastructural level core filaments are present in the microvilli associated with intestinal metaplasia and the glandular pattern of gastric carcinoma. Microvilli with dense cores have also occasionally been described on the free surface (as well as bordering intracellular cysts) in individual tumour cells, using electron microscopy (Nevalainen and Jarvi 1977; Tatematsu et al. 1986). Mucus stains, enyzme histochemistry or Alcian PAS can be used to indicate whether cells phenotypically express mucins or other proteins or enzymes typical of intestine or stomach (Iida and Kusama 1982; Fiocca et al. 1987; Tatematsu et al. 1986; Sasano et al. 1969; Santini 1987).

Our results, using cytoskeletal proteins and intestinal hydrolases as markers, confirm that intestinal metaplasia is characterized by markers usually associated with the small intestine. They further suggest that while normal gastric epithelium and normal intestinal epithelium can be represented by two defined and invariant sets of marker molecules some of which are described here, the spectrum of markers displayed in and on signet ring carcinoma cells is more variable. It may reflect either gastric differentiation, intestinal differentiation or a pattern lying somewhere between the two. A similar idea is apparent in the classification scheme for signet ring carcinomas proposed by Tatematsu et al. 1986 on grounds of differential mucin expression.

Diagnostic applications

There are only a very few keratin antibodies which work well on tissue routinely fixed in formaldehyde and embedded in paraffin. In the current study both KL1, (Viac et al. 1983) and CK8, an antibody specific for the keratin 8 polypeptide (Osborn et al. 1986) stained both intestinal and diffuse gastric carcinomas fixed in this way. Examination of Table 2 shows that staining was somewhat dependent on storage time. A decrease in immunoreactivity with time has also been noted with some, but not all, gastrointestinal carcinomas originally fixed in alcohol and embedded in paraffin (Osborn, unpublished results). Thus reactivity on routinely processed specimens with a particular antibody may depend not only on the age of the sample, but also on how it has been fixed, stored and treated prior to staining. In addition the amount of keratin and the particular keratin polypeptides originally present may be important.

Although gastric carcinomas of the intestinal type can be easily identified in histological specimens using conventional stains, some gastric carcinomas of the diffuse type can be more difficult to identify. This is particularly true if only a few tumour cells which lack the characteristic signet ring shape are present. In such cases an immunohistological technique which positively identifies tumour cells may be of advantage (Fig. 2, Altmannsberger et al. 1982). For such purposes a keratin specific antibody is probably more useful than a villin antibody. This is first because the requirements for fixation are less rigorous and second because while all signet ring cell carcinoma cells are uniformly keratin positive when tested with a broad specificity keratin antibody only some are villin positive. However the use of a palette of antibodies such as those described here may in time allow a more careful characterization of the various cell types comprising gastric carcinomas. Finally the finding of some keratin 7 positive gastric carcinomas has to be taken into account when using keratin specific antibodies in the differential diagnosis of gastrointestinal carcinomas (c.f. Osborn et al. 1986).

Acknowledgement. We thank Susanne Isenberg for expert technical assistance. Dr. Hans-Peter Hauri very kindly provided the antibodies to aminopeptidase N and sucrase isomaltase. This work was supported in part by a grant from the Deutsche Krebshilfe to M.O.

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Accepted May 19, 1988