Virchows Arch (1997) 431:159–166

ORIGINAL ARTICLE

Yi Cao · Peter M. Schlag · Uwe Karsten

Immunodetection of epithelial mucin (MUC1, MUC3) and mucin-associated glycotopes (TF, Tn, and sialosyl-Tn) in benign and malignant lesions of colonic epithelium: apolar localization corresponds to malignant transformation

Received: 18 February 1997 / Accepted: 2 April 1997

Abstract Epithelial mucins are present at the apical membranes of gastrointestinal epithelial cells or in their secretions. In this study, we examined the occurrence of peptide epitopes of the mucins MUC1 and MUC3 and of three mucin-associated glycotopes (TF, Tn, and s-Tn) in a series of colorectal tissue samples (normal colon, adenomas with different grades of dysplasia, carcinoma in situ, and invasive carcinomas). A new monoclonal antibody to a conformation-dependent peptide epitope of MUC1 was employed, which does not react with the fully glycosylated mucin as found in normal gastrointestinal mucosa. We found that adenomas acquired the ability to expose Tn, s-Tn, TF and MUC1 epitopes, and this correlated with increasing malignant potential. The secretory mucin, MUC3, revealed a different pattern: it was detectable in all sections, with maximum expression in adenomas and decrease in carcinomas. Most importantly, normal mucosa and benign lesions showed supranuclear and/or apical distribution of these antigens, but malignant lesions and lesions with a very high risk of malignancy revealed diffuse cytoplasmic and basolateral membrane localization. The immunohistological response to a combination of MUC1-related antibodies may assist in assessing the malignant potential and status of lesions of the colon.

Key words Mucin · Thomsen-Friedenreich antigen · Carbohydrate · Carcinogenesis · Colon tumour

Introduction

Models of tumour progression have been proposed in which the accumulation of genetic changes is assumed to be responsible for successive waves of clonal expansion of transformed cells [2, 10]. Alterations of glycoconjugates on the cell surface during the development of colorectal tumours have not been studied systematically to a similar extent. Over the past decade mucins and mucin-associated carbohydrate structures, which are major components of apical membranes of epithelial cells, have been found to undergo drastic changes on malignant tumours, and they are thought to contribute to their invasive and metastatic growth [5, 7, 13]. Recently, a number of important functional aspects have been attributed to mucins and mucin-associated carbohydrate structures: overexpression of the mucin in carcinoma cells with the consequence of strongly reduced intercellular adhesion and integrin- and E-cadherin-mediated cell-cell interactions [20, 32]; mucin and mucin-associated glycan-mediated adherence to basement membrane and matrix proteins [28]; and binding of the DF3/MUC1 cytoplasmic tail to the Grb2 protein [25]. Therefore, mucins should be seen as a group of biologically active molecules, which may be involved in the fundamental functional changes that transform an ordinary epithelial cell into a carcinoma cell, as well as in the process of tumour progression. Moreover, glycosylation changes may provide diagnostically useful markers.

We examined normal, premalignant and malignant colonic tissues for the expression of mucin core proteins (MUC1 and MUC3) and for the presence of mucin-associated carbohydrate structures, Thomsen-Friedenreich antigen (TF, Galβ1-3GalNAcα1-*O*-Ser/Thr), Tn (GalNAcα1-*O*-Ser/Thr), and sialosyl-Tn (s-Tn, NeuAcα2-6GalNAcα1-*O*-Ser/Thr) in various stages of tumour development. We also intended to analyse the simultaneous expression of these biosynthetically linked structures with respect to their mutual relationships.

Y. Cao · U. Karsten (✉)
Max Delbrück Centre for Molecular Medicine,
D-13125 Berlin-Buch, Germany
Tel.: (49)30-94062863, Fax: (49)30-94062101

P.M. Schlag
Robert Rössle Clinic, Rudolf-Virchow-Klinikum,
Humboldt University, Berlin-Buch, Germany

Materials and methods

Primary colorectal carcinoma tissues were obtained from 74 patients undergoing surgical resection. The 23 adenomatous polyps were removed by endoscopic polypectomy and surgical resection.

The 17 normal colonic mucosae included 4 cases taken at autopsy from individuals without colorectal malignancies and 13 specimens from patients with cancer taken at surgery or colonoscopy from apparently normal parts of the colonic wall distant from the tumour. These 17 mucosae showed normal histomorphologic and cytomorphologic appearances. All specimens except 4 cases of adenomatous polyps were immediately frozen and stored at −80° C. Cryosections were cut 4 μm thick. The remaining 4 cases of adenomatous polyps were fixed in formalin, embedded in paraffin and cut into 4-μm serial sections.

Classification of primary carcinomas was done on haematoxylin-eosin stained sections according to UICC and World Health Organization recommendations [11, 18]. T stage and lymph node metastases were recorded on the basis of the pathological examinations. Haematogenous metastasis was defined according to a clinical follow-up for at least 1 year. Adenomatous polyps were classified as to histological type and grade of dysplasia according to the criteria of Konishi and Morson [21]. Cases in which adenomas contained carcinoma in situ (malignant cells confined to the mucosa) were grouped with those containing severe dysplasia.

A76-A/C7 is a newly developed anti-MUC1 antibody from this laboratory (manuscript in preparation). Briefly, this hybridoma cell line was generated by fusion of spleen cells from a Balb/c mouse, immunized with life T-47D (breast carcinoma) cells after treatment with neuraminidase from *V. cholerae* with X63-Ag8.653 plasmocytoma cells. Antibody screening was done by cytochemistry (immunofluorescence tests) with T-47D cells. After cloning, antibody specificity was determined by immunohistochemistry with normal and malignant human tissues, immunocytochemistry with MUC1-positive and -negative cell lines, and enzyme-linked immunosorbent assays (ELISAs) with a variety of glycoproteins and peptides, among them human milk fat globule membranes and synthetic MUC1 peptides of various lengths. The antibody A76-A/C7 (IgG1, κ) recognizes a peptide epitope of the MUC1 tandem repeat which is conformation-dependent (and therefore requires a minimal length of the peptide [>3 tandem repeats] for binding in ELISA). For comparison, the anti-MUC1 antibody HMFG-2 (IgG1) was used, which recognizes the epitope DTR [4]; it was purchased from Dianova, Hamburg, Germany. The anti-TF mAb A78-G/A7 (IgM) has been described elsewhere [19]. M3.2 (anti-MUC3, IgG2 [1]) and 12A8-C7-F5 (anti-Tn, IgM [30]) were kindly provided by Dr. P.-X. Xing (Heidelberg, Victoria, Australia) and Prof. Dr. G.F. Springer (Chicago, USA), respectively. B72.3 (anti-s-Tn, IgG1) was purchased from Biogenesis (Bournemouth, UK).

Staining of tissue sections was performed by the avidin-biotin-peroxidase complex (ABC) method with a commercial kit (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, Calif.) as follows. Paraffin sections were deparaffinized, and frozen sections were air-dried at room temperature (RT), followed by fixation with 10% formalin in PBS for 15 min at RT. Endogenous peroxidase activity was eliminated by treatment with 3% H_2O_2 in PBS for 30 min at RT. Nonspecific binding sites were blocked with normal rabbit serum that had been absorbed with neuraminidase-treated red blood cells from blood group 0 individuals. After washing with PBS, sections were incubated with mAbs in appropriate dilutions for 1 h at RT. The thoroughly washed sections were treated with biotinylated anti-mouse immunoglobulin antiserum for 30 min at RT, and thereafter with the ABC complex. Colour development during incubation with the peroxidase substrate (diaminobenzidine, DAB, Sigma, Deisenhofen, Germany) was controlled under the microscope with a positive control section. Double staining was done in 5 adenomas and 10 carcinomas. Briefly, sections were incubated with first mAbs, then the thoroughly washed sections were treated with rabbit anti-mouse immunoglobulin and APAAP (alkaline phosphatase anti-alkaline phosphatase complex, Biogenesis), and colour development was accomplished with the DAKO Fast Red Substrate System (DAKO, Copenhagen, Denmark); thereafter, the second mAb was added, followed by biotinylated anti-mouse IgG (γ-chain) or anti-mouse IgM (μ-chain; Sigma) and the ABC complex as above. For successful double staining the first and second mAbs must belong to different Ig classes. Counterstaining was done with haematoxylin. Negative controls for single or double staining were incubated with a comparable dilution of either IgG or IgM from a mouse plasmocytoma (Sigma) instead of the mAb.

Sections from all tissues were treated with the antibodies A76-A/C7, HMFG-2, A78-G/A7, 12A8-C7-F5, and B72.3. MAb M3.2 was employed in all normal colonic mucosae and adenomatous polyps, and in 32 cases of carcinomas. Four mAbs were used for double staining in the following combinations: HMFG-2 and A78-G/A7; HMFG-2 and 12A8-C7-F5; A78-G/A7 and B72.3.

Scoring was performed as follows: – all cells negative; + <30% positive cells; 2+ 30–60% positive cells; 3+ >60% positive cells. The percentage of positive cells was estimated in several optical fields (12.5×lens).

Data were analysed with either the Chi-square test or Fisher's exact probability test. Correlation analysis of enumeration data was also performed [26]. The significance level (α) was 0.05.

Results

The frequencies and intensities of staining for MUC1, MUC3, TF, Tn and s-Tn in normal mucosa, adenomas, and carcinomas are shown in Fig. 1. Similar results obtained with HMFG-2 and A76-A/C7 were combined. Staining frequencies were compared among normal mucosa and adenomas, adenomas with mild, moderate and severe dysplasia, well, moderately and poorly differentiated adenocarcinomas, and different histological types of adenomas and carcinomas.

Three different patterns of antigen localization were observed in the tissues examined. The first was a supranuclear pattern, in which the antigens are localized only in the circumambient supranuclear portion (Golgi zone) of goblet and columnar cells (Fig. 2). In the apical expression pattern the antigens are present in the apical membranes of both goblet cells and columnar cells or in the apical cytoplasm of columnar cells and/or in vacuoles of goblet cells (Fig. 2A, B, D). The diffuse cytoplasm and basolateral membrane pattern showed antigens distributed diffusely throughout the cell or distinctly in the basolateral membrane (Fig. 2J–L). The frequencies of the three staining patterns in various colonic tissues are presented in Fig. 3. In adenomas, the patterns of antigen localization correlated with the morphological feature of epithelial cells. Polar epithelial cells showed the supranuclear pattern or the apical expression pattern. Cells that had lost polarity exhibited the diffuse cytoplasm and basolateral membrane staining pattern. In malignant lesions with positive staining we found that almost all positively staining carcinomas showed the third pattern, although not necessarily in all cells of a given tumour. Generally, in glandular adenocarcinomas the antigens were detected predominantly at the apical membrane and in apical parts of the cytoplasm. Diffuse cytoplasm or basolateral membrane patterns were also seen. In nongland-forming areas of tumours and in invasive cells, the diffuse cytoplasm or basolateral membrane pattern was observed in almost all positive cells.

The simultaneous expression of MUC1, MUC3, TF, Tn and s-Tn in the same tissues was examined. In general, we observed a correlation between the four antigens

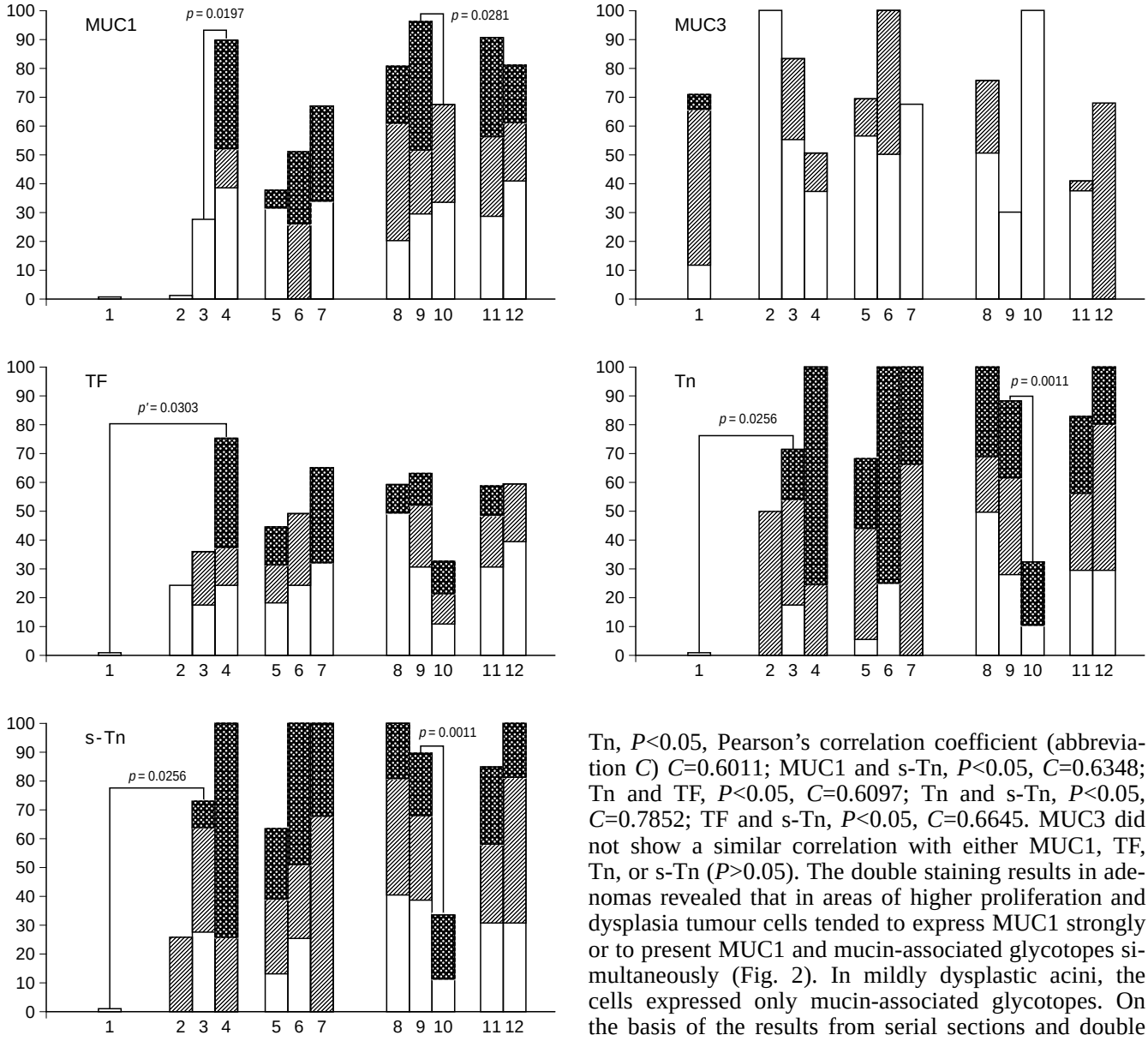
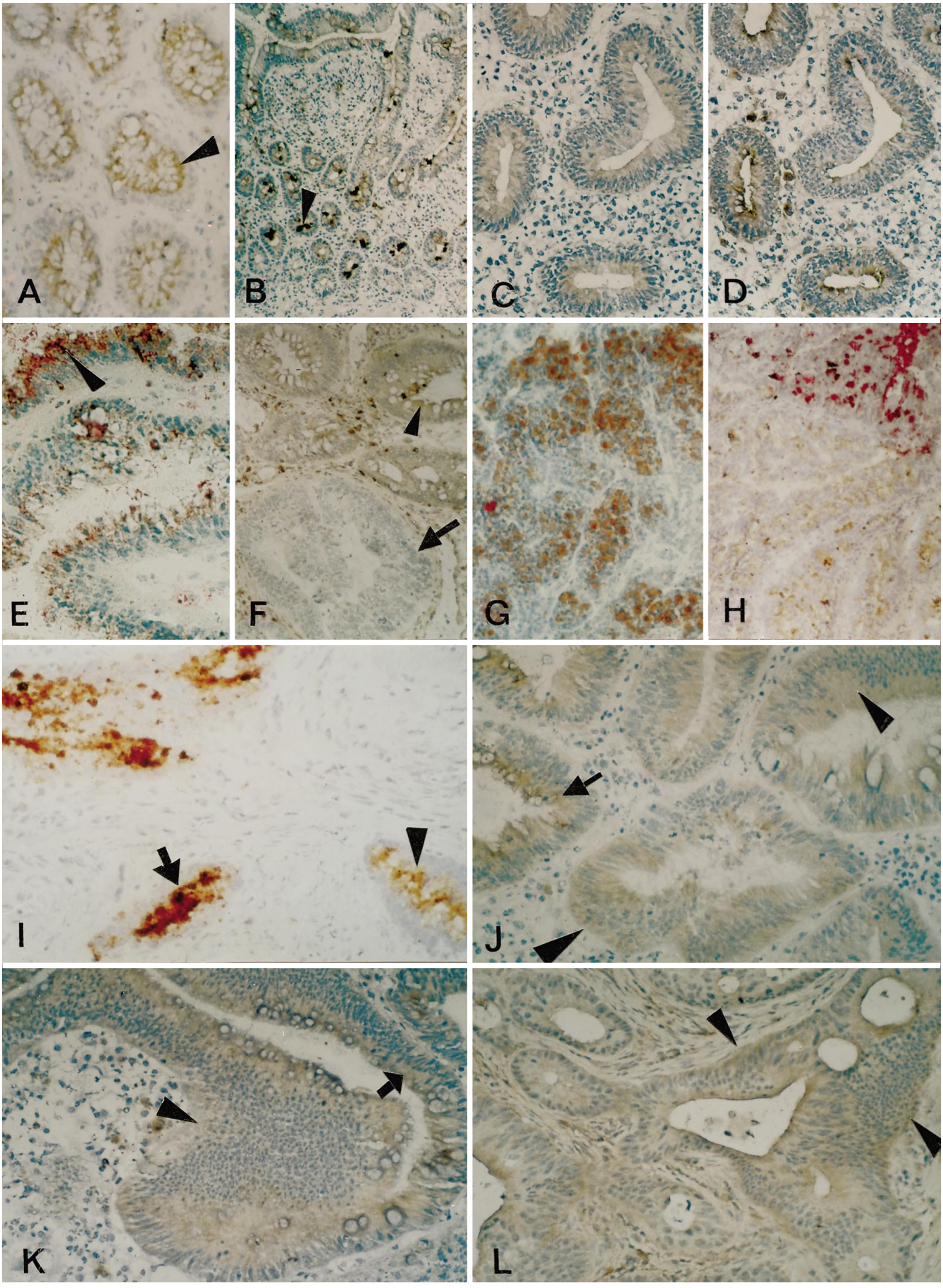


Fig. 1 Comparison of the frequencies (percentage of positive cases) of anti-MUC1, anti-MUC3, anti-TF, anti-Tn and anti-sialosyl (s)-Tn staining normal, adenomatous and cancerous tissues of colon (*1* normal colon, *2–7* adenomatous polyps: *2* mild dysplasia, *3* moderate dysplasia, *4* severe dysplasia or carcinoma in situ, *5* tubular adenoma, *6* tubulovillous adenoma, *7* villous adenoma, *8–12* primary colorectal carcinoma: *8* well-differentiated adenocarcinoma, *9* moderately differentiated adenocarcinoma, *10* poorly differentiated adenocarcinoma, *11* adenocarcinoma, *12* mucinous adenocarcinoma; □ <30% reactive cells, ▨ 30–60% reactive cells, ▩ >60% reactive cells). Statistically significant differences ($P<0.05$, p' one-sided test) are indicated, with the exception of the panel for MUC1, where column *4* is not only significantly different from *3*, but also from columns *1* and *2*

(MUC1, TF, Tn and s-Tn) with respect to the intensity of staining in most cases of adenocarcinomas (tumours strongly positive for one of the antigens were also intensely stained for the others). Correlation analysis revealed the following: MUC1 and TF, $P>0.05$; MUC1 and Tn, $P<0.05$, Pearson's correlation coefficient (abbreviation *C*) $C=0.6011$; MUC1 and s-Tn, $P<0.05$, $C=0.6348$; Tn and TF, $P<0.05$, $C=0.6097$; Tn and s-Tn, $P<0.05$, $C=0.7852$; TF and s-Tn, $P<0.05$, $C=0.6645$. MUC3 did not show a similar correlation with either MUC1, TF, Tn, or s-Tn ($P>0.05$). The double staining results in adenomas revealed that in areas of higher proliferation and dysplasia tumour cells tended to express MUC1 strongly or to present MUC1 and mucin-associated glycotopes simultaneously (Fig. 2). In mildly dysplastic acini, the cells expressed only mucin-associated glycotopes. On the basis of the results from serial sections and double staining too, we conclude that in adenomas, mildly and moderately dysplastic lesions show simultaneous expression of MUC3, TF, Tn and s-Tn, and lack of MUC1 in most cases. In some severely dysplastic lesions MUC1 became positive, whereas MUC3 expression decreased slightly. In adenocarcinomas, the majority of cases showed a correlation between the four antigens (MUC1, TF, Tn and s-Tn) with respect to the intensity of staining (excluding MUC3). The extent of mucin and mucin-associated glycotope presentation was ranked according to the frequency and intensity of positive staining in the study as follows. In all adenomas, regardless of their degree of dysplasia, the sequence was Tn>s-Tn>TF> MUC1; in adenocarcinomas, MUC1 moved to the first position. In contrast to MUC1, MUC3 was expressed in normal colon, benign lesions, and mucinous carcinomas at higher frequencies, and to a lesser extent in adenocarcinomas. In mucinous carcinomas the mucus itself did not react with the anti-MUC3 mAb.


A
B
C
D
E
F
G
H
I
J
K
L

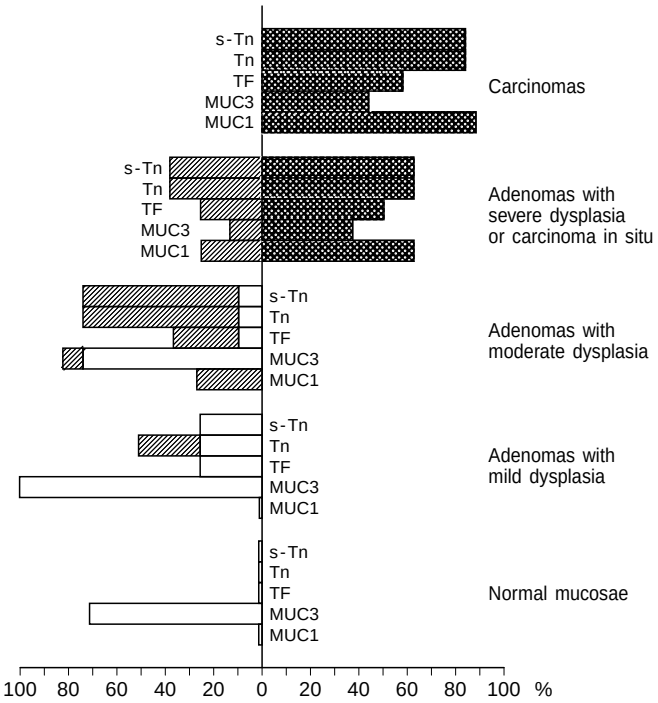


Fig. 3 Percentage of the different staining patterns of anti-MUC1, anti-MUC3-, anti-TF, anti-Tn and anti-s-Tn in normal, adenomatous and carcinomatous areas of colon (□ supranuclear pattern, ▨ apical expression pattern, ▩ diffuse cytoplasm and basolateral membrane pattern)

No significant correlations were found between the presence of these antigens and T stage, or lymph node status. However, we observed a significantly higher expression rate of MUC1 and TF in carcinomas from patients with blood-borne metastasis than in patients without metastases.

◀ **Fig. 2A–L** Immunohistochemical analysis of mucin core peptides and mucin-associated glycotopes in colorectal adenomas and carcinomas. **A** Adenoma with mild dysplasia, supranuclear region (*arrowheads*) of epithelial cells stained with 12A8-C7-F5 (anti-Tn). **B** Adenoma with mild dysplasia, positive in goblet cells' vacuoles (*arrowhead*) with B72.3 (anti-s-Tn). **C, D** Serial sections of an adenoma with moderate dysplasia, negative for A76-A/C7 (anti-MUC1, **C**) but positive at the apical membrane with A78-G/A7 (anti-TF, **D**). **E** Adenomas with severe dysplasia double stained with 12A8-C7-F5 (*brown*) and HMFG-2 (anti-MUC1, *red*). [Note: some tumour cells (*arrowhead*) show mixed colour.] **F** Carcinoma stained with M3.2 (anti-MUC3). [Note: tumour cells (*arrow*) are negative for M3.2, while the adjacent mucosa (*arrowhead*) is stained.] **G** Carcinoma double stained with A78-G/A7 (*brown*) and B72.3 (*red*). (Note: most tumour cells show mixed colour.) **H** Carcinoma double stained with A78-G/A7 (*brown*) and HMFG-2 (*red*). **I** Carcinoma double stained with 12A8-C7-F5 (*brown*) and HMFG-2 (*red*). [Note: tumour cells show mixed colour, either stronger in *red* (*arrow*), or in *brown* (*arrowhead*).] **J** Adenomas with severe dysplasia, MUC1 labelled with A76-A/C7 showing the apical expression pattern (*arrow*) or the diffuse cytoplasm and basolateral membrane pattern (*arrowhead*). **K** Adenomas with severe dysplasia, TF detected with A78-G/A7 revealing either the apical expression pattern (*arrow*) or the diffuse cytoplasm and basolateral membrane pattern (*arrowhead*). **L** Carcinoma, MUC3 labelled with M3.2, showing the diffuse cytoplasm and basolateral membrane pattern (*arrowheads*)

Discussion

The concept of tumourigenesis as a multistep process is widely accepted. The various stages of colorectal tumour development provide an excellent model to study genetic, cellular, biochemical, and immunological alterations occurring during this process, and to assess their importance and possible functional role during malignant transformation. In the past decade many studies have focused on the changes of genes and chromosomes [2, 10]. Mucins are major components present at the surface membranes of gastrointestinal epithelial cells and in their secretions. They are thought to act as a barrier separating these cells from the action of luminal degradative enzymes, particularly proteases. In colorectal carcinomas and premalignant lesions, alterations of mucin expression and glycosylation have been described [5, 7, 13, 14, 17, 34, 35], rendering these mucins, and especially the membrane-bound MUC1, highly interesting molecule for cancer diagnosis and therapy. From a separate set of experiments (Y. Cao et al., unpublished work) we now know that, in contrast to earlier observations, MUC1 is expressed both in normal and in malignant colon epithelium, although with differences in its glycosylation, which alters the immunoreactivity of the molecule. Our MUC1 mAb employed here, A76-A/C7, does not stain the normally glycosylated MUC1 of colorectal epithelia. This antibody recongizes a conformation-dependent peptide epitope which in the gastrointestinal tract is tumour specific. It was, therefore, of interest to determine at what stage of the colon tumourigenesis this epitope first appears. This was compared with the appearance of MUC1-associated, tumour-related glycotopes, TF, Tn and s-Tn, and a secretory gastrointestinal mucin, MUC3. For this pupose we have used a series of colorectal tissue samples (normal colon, adenomas with different grades of dysplasia, carcinoma in situ, invasive carcinoma) and have compared the simultaneous presence of all epitopes examined by parallel staining of serial sections and double staining, resulting in the most comprehensive study of this kind published so far. The results are summarized in Fig. 4.

Normal mucosae were negative for the A76-A/C7 and HMFG-2 epitopes of MUC1, TF, Tn and s-Tn. In adenomas, we observed a gradual increase of expression of these antigens from adenomas with mild dysplasia through adenomas with moderate dysplasia to adenomas with severe dysplasia, in agreement with previous studies dealing with partial aspects [17, 34, 35]. In addition, these antigens showed a tendency to be more abundant in villous adenomas. It is obvious that the grade of dysplasia of adenomas is closely related to their size; adenomas that are larger, of higher grades of dysplasia, or of the villous type have a higher malignant potential [21]. Well-differentiated and moderately differentiated carcinomas exhibited MUC1, TF, Tn and s-Tn at higher frequencies than did poorly differentiated carcinomas. Thus, the alterations in glycosylation as exemplified by the appearance of MUC1, TF, Tn and s-Tn are early events in neo-

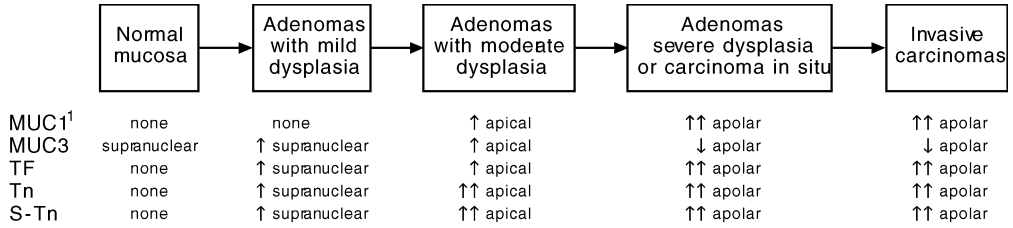


Fig. 4 A model of the alterations of mucin and mucin-associated glycotopes during colorectal tumorigenesis (*none* no expression, ↑ increased expression, ↑↑ strongly increased expression, ↓ decreased expression, *apical* antigen distribution in the apical membranes or apical cytoplasm of epithelial cells, *supranuclear* antigen distribution in the circumambient supranuclear portion of epithelial cells, *apolar* antigen distribution diffusely in the cytoplasm and/or at the basolateral membrane of epithelial cells. [1] A76-A/C7 epitope

plastic transformation and are sometimes no longer visible in the fully transformed, poorly differentiated state; importantly, enhanced levels of MUC1, TF, Tn and s-Tn in adenomas seem to be associated with their malignant potential. According to our results, adenomas with severe dysplasia, which carry a high cancer risk, and moderately and well differentiated carcinomas, which means the vast majority of cases of colorectal adenocarcinomas [8], expressed MUC1, TF, Tn and s-Tn in the highest frequency and intensity. This suggests a possible diagnostic application through the examination of these antigens in tissues, sera and secretions. The expression pattern of MUC3 was clearly distinct from that of MUC1 and the mucin-associated glycotopes examined. In the normal mucosa, epithelial cells presented MUC3 in the supranuclear region. In adenomas with mild and moderate dysplasia, the expression of MUC3 was increased in regions of altered architecture in a number of cases, and its presence in vacuoles of goblet cells was also occasionally observed. In severe dysplasia and malignant lesions, which were made up mainly of nonmucinous columnar cells, the percentage of MUC3-positive cells was slightly lower. This result differs from that of a recent study employing another anti-MUC3 antiserum, M3P [14]. In this study, tubulovillous and severely dysplastic adenomas, and carcinomas showed significantly stronger staining than normal mucosa, despite a lower mRNA level in these neoplastic cells. The differences may be due to the fact that the epitope of M3.2 differs from that of M3P.

The changes in the cellular distribution of MUC1, MUC3, TF, Tn and s-Tn observed during the process of malignant transformation seem important. Three types of cellular localization of mucin and mucin-associated glycotope in distinct phases of cancerogenesis could be distinguished as a *supranuclear* pattern, which was present in normal mucosae and in adenomas with mild dysplasia; examples were MUC3 in normal mucosa, or TF, Tn and s-Tn in adenomas with mild dysplasia, and a similar pattern was seen in the small intestine [6]. The *apical membrane and cytoplasm* pattern was seen in various adenomas and carcinomas. The *diffuse cytoplasm and basolateral membrane* pattern was seen only in adenomas with severe dysplasia and in carcinomas. These different patterns may be explained on the basis of mucin biosynthesis and intracellular transport: epithelial cells with the supranuclear pattern are most likely to be able to fully accomplish glycosylation and apical transport, whereby only intermediates in the Golgi can be visualized. This interpretation implies that full glycosylation may lead to masking of mucin peptide epitopes (Y. Cao et al., unpublished observation). Lesions with the second pattern probably synthesize incompletely glycosylated mucins, but their polarized localization is essentially retained. Lesions in which the antigens are diffusely or basolaterally distributed have lost the capacity both for complete glycosylation and for apical transport, resulting in mucin accumulation in the whole cells and/or its apolar distribution. This phenomenon is also observed in other carcinomas, such as breast and ovarian carcinomas [22, 31]. Histochemistry alone cannot decide whether the observed loss of polar expression of mucin plays an active part in the process of carcinogenesis. However, one or more of the following mechanisms may explain the phenomenon. Mucins at the basolateral membrane may reduce the adherence of epithelial cells to their neighbouring cells [32], since incompletely glycosylated, basolaterally distributed mucin might allow binding to fibronectin, laminin and/or collagen type IV of the basal membrane and the extracellular matrix [28] and possibly also to galectins. Mucin localized anywhere on the cell surface may shield the cell from lower pH caused by high glycolytic activity [3] or mucins more or less uniformly distributed at the surface might protect tumour cells from the immune system [12]. Since the cytoplasmic domain of MUC1 is associated with the ras signal system [25], it is also possible that an abnormal distribution of the mucin interferes with normal cellular signalling. Finally, the mucin-associated, newly exposed glycotopes (eg TF, sialosyl-Le^X) at the cellular surface adhere to receptors on endothelial cells or liver cells, respectively, and are thus involved in metastasis [5, 16]. It is tempting to speculate that incomplete glycosylation of mucin may be an early step in the transformation of a normal epithelial cell, and that this mucin may be subject to an abnormal apolar distribution with more serious alteration. This may be a key step in their pathophysiological function, leading to further consequences such as causing or promoting the epithelial cells to leave their original site and break through the basement membrane, thus starting invasive growth. In our opinion, the apolar distribution of mucin and mucin-associated glycotopes is

a decisive sign of malignant transformation in histopathology. We also noted that cells with apolar mucin distribution showed an apolar arrangement in morphologic architecture.

Thomsen-Friedenreich-related glycotopes are known to be carried by MUC1 in mammary carcinomas [15]. In colorectal carcinomas, this has not yet been explicitly examined. In our study, the expression of the A76-A/C7 epitope, indicating incompletely glycosylated MUC1, correlated strongly with that of the glycotopes examined. This indicates that MUC1 is a major carrier molecule of TF, Tn, and s-Tn in colorectal adenomas and carcinomas. MUC3 also seems to be a carrier molecule for these glycotopes, especially in mucinous carcinoma. Previous studies have indicated that s-Tn(+) tumour cells are negative for TF [9, 24, 33]. Biochemical studies strongly suggest that galactose cannot be transferred to sialic acid-containing oligosaccharides [27]. However, in our double staining experiments many tumour cells presented simultaneously with both TF and s-Tn. We were surprised by this finding, which implicates the concomitant accumulation of competing intermediates of biosynthesis. However, a substantial number of tumour cells within a given tumour did not express all antigens simultaneously, as was very clearly seen in our serial and double staining experiments. These cancer cells show the complementary expression of these antigens. (There are cells expressing some antigens and lacking others, but the latter can be found on another subset of cells.) This indicates that in clinical settings the employment of a combination of several antigens as tumour markers is essential to improve diagnostic sensitivity and therapeutic targeting.

Our finding of MUC1 expression in a significantly higher percentage of primary tumours from patients with blood-borne metastases than of tumours from patients without metastasis supports a previous study [23]. This might be explained by one or more of the above-mentioned functions of mucin on the surface of tumour cells. TF expression shows similar correlations to MUC1 with blood-borne metastasis. This can be explained by the fact that TF permits tumour cell binding to asialoglycoprotein receptors on hepatocytes known to be involved in the development of liver metastasis [5, 29].

In summary, during tumourigenesis of colon epithelia, mucins show characteristic changes of glycosylation and distribution (Fig. 4). When these changes reach a certain degree, they may cause or promote invasive growth. Clearly, genetic changes are primary events in malignant transformation and tumour progression, but abnormal glycosylation, and particularly the nonapical distribution of glycoconjugates at the cellular surface, may play an additional active part in these processes. On the other hand, MUC1, TF, Tn and s-Tn can be considered to be reliable tumour markers in clinical diagnosis and therapy, especially in a combined approach.

Acknowledgements We thank Prof. G.F. Springer (Chicago) and Dr. P.-X. Xing (Heidelberg, Australia) for providing monoclonal antibodies, and Prof. P. Stosiek (Cottbus, Germany) for a number of normal colon specimens. We especially acknowledge the help of Dr. M. Ghadimi (Tumour Bank, MDC, Berlin, Germany) who collected and provided most of the samples. This work was supported by the Deutsche Forschungsgemeinschaft (grant Ka 921/1).

References

1. Apostolopoulos V, Xing PX, McKenzie IFC (1995) Anti-peptide monoclonal antibodies to intestinal mucin 3. J Gastroenterol Hepatol 10:555–561
2. Boland CR, Sato J, Appelman HD, Bresalier RS, Feinberg AP (1995) Microallelotyping defines the sequence and tempo of allelic losses at tumour suppressor gene loci during colorectal cancer progression. Nat Med 1:902–909
3. Bramwell ME, Bhavanandan VP, Wiseman G, Harris H (1983) Structure and function of the Ca antigen. Br J Cancer 48:177–183
4. Burchell J, Taylor-Papadimitriou J, Boshell M, Gendler S, Duhig T (1989) A short sequence, within the amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes. Int J Cancer 44:691–696
5. Cao Y, Karsten U, Liebrich W, Haensch W, Springer GF, Schlag P (1995) Expression of Thomsen-Friedenreich-related antigens in primary and metastatic colorectal carcinomas: a reevaluation. Cancer 76:1700–1708
6. Cao Y, Stosiek P, Springer GF, Karsten U (1996) Thomsen-Friedenreich-related carbohydrate antigens in normal adult human tissues: a systematic and comparative study. Histochem Cell Biol 106:197–207
7. Carrato C, Balague C, Bolos CD, Gonzalez E, Gambus G, Planas J, Perini JM, Andreu D, Real FX (1994) Differential apomucin expression in normal and neoplastic human gastrointestinal tissues. Gastroenterology 107:160–172
8. Carriaga MT, Hensom DE (1995) The histologic grading of cancer. Cancer 75:406–421
9. Chung YS, Yamashita Y, Kato Y, Nakata B, Sawada T, Sowa M (1996) Prognostic significance of T antigen expression in patients with gastric carcinoma. Cancer 77:1768–1773
10. Fearon ER, Vogelstein BA (1990) Genetic model for colorectal tumorigenesis. Cell 61:759–767
11. Hermanek P, Scheibe O, Spiessl B, Wagner G (1992) TNM Classification of malignant tumours, 4th edn. Springer, Berlin Heidelberg New York
12. Hilkens J, Vos HL, Wesseling J, Boer M, Storm J, Van der Valk S, Calafat J, Atriarca C (1995) Is episialin/MUC1 involved in breast cancer progression? Cancer Lett 90:27–33
13. Ho SB, Niehans GA, Lyftogt C, Yan PS, Cherwitz DL, Gum ET, Dahiya R, Kim YS (1993) Heterogeneity of mucin gene expression in normal and neoplastic tissues. Cancer Res 53:641–651
14. Ho SB, Ewing SL, Montgomery CK, Kim YS (1996) Altered mucin core peptide immunoreactivity in the colon polyp-carcinoma sequence. Oncol Res 8:53–61
15. Hull SR, Bright A, Carraway KL, Abe M, Hayes DF, Kufe DW (1989) Oligosaccharide differences in the DF3 sialomucin antigen from normal human milk and the BT-20 human breast carcinoma cell line. Cancer Commun 1:261–267
16. Irimura T, Nakamori S, Matsushita Y, Taniuchi Y, Todoroki N, Tsuji T, Izumi Y, Kawamura Y, Hoff SD, Cleary KR, Ota DM (1993) Colorectal cancer metastasis determined by carbohydrate-mediated cell adhesion: role of sialyl-Le^x antigens. Semin Cancer Biol 4:319–324
17. Itzkowitz SH, Bloom EJ, Lau TS, Kim YS (1992) Mucin associated Tn and sialosyl-Tn antigen expression in colorectal polyps. Gut 33:518–523
18. Jass JR, Sobin LH (1989) Histological typing of intstinal tumours, 2nd edn. Springer, Berlin Heidelberg New York
19. Karsten U, Butschak G, Cao Y, Goletz S, Hanisch FG (1995) A new monoclonal antibody (A78-G/A7) to the Thomsen-Friedenreich pan-tumor antigen. Hybridoma 14:37–44

20. Kemperman H, Wijnanda Y, Wesseling J, Niessen CM, Sonnenberg A, Roos E (1994) The mucin epiglycanin on TA3/Ha carcinoma cells prevents $\alpha_6\beta_4$-mediated adhesion to laminin and kalinin and E-cadherin-mediated cell-cell interaction. J Cell Biol 127:2071–2080
21. Konishi F, Morson BC (1982) Pathology of colorectal adenomas: a colonoscopic survey. J Clin Pathol 35:830–841
22. McGuckin MA, Walsh MD, Hohn BG, Ward BG, Wright RG (1995) Prognostic significance of muc1 epithelial mucin expression in breast cancer. Hum Pathol 26:432–439
23. Nakamori S, Ota DM, Clearly KR, Shirotani K, Irimura T (1994) MUC1 mucin expression as a marker of progression and metastasis of human colotectal carcinoma. Gastroenterology 106:353–361
24. Ogata S, Chen A, Itzkowitz SH (1994) Use of model cell lines to study the biosynthesis and biological role of cancer-associated sialosyl-Tn antigen. Cancer Res 54:4036–4044
25. Pandey P, Kharbanda S, Kuff D (1995) Association of the DF3/MUC1 breast cancer antigen with Grb2 and the Sos/Ras exchange protein. Cancer Res 55:4000–4003
26. Sachs L (1992) Angewandte Statistik, 7th edn. Springer, Berlin Heidelberg New York
27. Schachter H, Mcguire EJ, Roseman S (1971) Sialic acids: XIII. A uridine diphosphate *d*-galactose: mucin galactosyltransferase from porcine submaxillary gland. J Biol Chem 246:5321–5328
28. Schwartz B, Bresalier RS, Kim YS (1992) The role of mucin in colon-cancer metastasis. Int J Cancer 52:60–65
29. Springer GF, Cheingsong-Popov R, Schirrmacher V, Desai PR, Tegtmeyer H (1983) Proposed molecular basis of murine tumor cell-hepatocyte interaction. J Biol Chem 258:5702–5706
30. Springer GF, Chandrasekaran EV, Desai PR, Tegtmeyer H (1988) Blood group Tn-active macromolecules from human carcinomas and erythrocytes: characterization of and specific reactivity with mono- and poly-clonal anti-Tn antibodies induced by various immunogens. Carbohydr Res 178:271–292
31. Tashiro Y, Yonezawa S, Kim YS, Sato E (1994) Immunohistochemical study of mucin carbohydrates and core proteins in human ovarian tumors. Hum Pathol 25:364–372
32. Wesseling J, Valk SW, Vos HL, Sonnenberg A, Hilkens J (1995) Episialin (MUC1) overexpression inhibits integrin-mediated cell adhesion to extracellular matrix components. J Cell Biol 129:255–265
33. Yamashita Y, Chung YS, Horie R, Kannagi R, Sowa M (1995) Alterations in gastric mucin with malignant transformation: novel pathway for mucin synthesis. J Natl Cancer Inst 87:441–446
34. Yuan M, Itzkowitz SH, Boland CR, Kim YD, Tomita JT, Palekar A, Bennington JL, Trump BF, Kim YS (1986) Comparison of T-antigen expression in normal, premalignant, and malignant human colonic tissue using lectin and antibody immunohistochemistry. Cancer Res 46:4841–4847
35. Zotter S, Lossnitzer A, Hageman PC, Delemarre JFM, Hilkens J, Hilgers J (1987) Immunohistochemical localization of the epithelial marker MAM-6 in invasive malignancies and highly dysplastic adenomas of the large intestine. Lab Invest 57:193–199

Note added in proof Pepscan analyses presented at the ISOBM TD-4 International Workshop on Monoclonal Antibodies against MUC1, San Diego 1996, have determined the A76-A/C7 epitope as APDTRP.