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Immunoreactivity of Lewis blood group and mucin peptide core antigens: Correlations with grade of dysplasia and malignant transformation in the colorectal adenoma-carcinoma sequence

S.E. Baldus[1], F.-G. Hanisch[2], C. Pütz[1], U. Flucke[3], S.P. Mönig[3], P.M. Schneider[3], J. Thiele[1], A.H. Hölscher[3] and H.P. Dienes[1]

[1]Institute of Pathology, [2]Center of Biochemistry and

[3]Department of Visceral and Vascular Surgery, University of Cologne, Cologne, Germany

Summary. Previous studies on the immunoreactivity of various mucin peptide and carbohydrate antigens in neoplastic colorectal tissues led to at least in part contradictory results. Therefore, we investigated a series of 42 adenomas and 44 carcinomas applying monoclonal antibodies (mabs) directed against Lewis blood group antigens (sialyl-Le^a, Le^x, sialyl-Le^x, Le^y) as well as mucin peptide cores (MUC1, MUC2 and MUC5AC) by immunohistochemistry. A statistically significant positive correlation between the development of high-grade dysplasia in colorectal adenomas and the immunoreactivity of Le^y and MUC1 epitopes was observed, whereas MUC2 exhibited a significant negative correlation. The reactivity of the other epitopes did not show an association with the progression of malignant transformation. Colorectal carcinomas were subdivided according to their histopathological subtype. The immunohistochemical staining resulted in a significantly stronger MUC2 reactivity of mucinous vs. tubular adenocarcinomas. Immunoreactivity of the MUC1-specific mab, which does not react with the fully glycosylated peptide core, showed a statistically non-significant inverse tendency, whereas all carbohydrate antigens displayed a strong expression in both tumor subtypes. Furthermore, correlations between mucin peptide and carbohydrate epitope labelling were evaluated. Progression of the adenoma-carcinoma sequence was accompanied by an increase of Le^y as well as MUC1 antigen and an increase of all Lewis antigens compared to MUC2 immunoreactivity. On the other hand, mucinous carcinomas exhibited an inverse pattern. In conclusion, these results demonstrate that Le^y and MUC1 immunoreactivity correlate with malignant transformation in the colorectum, whereas MUC2 represents a marker for low-grade dysplasia and the subtype of mucinous carcinomas.

Key words: Lewis blood group antigens, Mucin antigens, Tumor antigens, Colon cancer, Monoclonal antibody (mab), Immunohistology

Offprint requests to: Dr. Stephan E. Baldus, Institute of Pathology, University of Cologne, Joseph-Stelzmann-Str. 9, 50924 Cologne, Germany. Fax: 0049-221-478-6360. e-mail: s-e.baldus@uni-koeln.de

Introduction

During the last years, various mucin-associated carbohydrate as well as peptide antigens expressed by colorectal neoplasms were investigated by immunohistochemistry. As has been reviewed comprehensively (Kim and Itzkowitz, 1988; Baldus & Hanisch, 2000), few studies included type 1 chain (Galß1-3GlcNAc) oligosaccharides, but most of them were focused on peripheral carbohydrate antigens based on type 2 chain (Galß1-4GlcNAc) backbones. The latter are represented by the monofucosylated Le^x and the difucosylated Le^y antigen. Le^x is expressed by colorectal adenomas as well as carcinomas independently from secretor status or localization, i.e. proximal vs. distal colon/rectum (Gong et al., 1985; Itzkowitz et al., 1986; Sakamoto et al., 1986; Baldus et al., 1995). Le^y, on the other hand, shows an ectopic, stronger reactivity in the distal colon and rectum (Brown et al., 1984; Bara et al., 1988; Baldus et al., 1995). Typically, Lewis blood group antigens may be modified by sialylation, which results into the formation of sialyl-Le^x (type 2 chain) or sialyl-Le^a (type 1 chain) oligosaccharides. Sialyl-Le^x is expressed by carcinomas, and low- and high-grade adenomas as well, but shows a stronger immunoreactivity in the latter (Hanski et al., 1990; Baldus et al., 1995). Sialyl-Le^a is also strongly reactive in colorectal carcinomas (Atkinson et al., 1982; Gong et

al., 1985), but its alterations during the adenoma-carcinoma sequence have not been thoroughly investigated up to now. All these oligosaccharides may be part of mucin glycoproteins, which have characteristic core peptide structures. These can be classified into membrane-bound (for example MUC1) and secretion-associated (for example MUC2 and MUC5AC) mucins. However, the former can also be found as secreted mucins (Baldus and Hanisch, 2000). In the past, MUC1 and MUC2 mucin peptide expression was immunohistochemically characterized. MUC1 was strongly expressed in tubular adenocarcinomas (Andrews et al., 1993; Ho et al., 1993; Baldus et al., 1998), and its binding in adenomas increased with the development of high-grade dysplasia (Cao et al., 1997; Baldus et al., 1998). MUC2, on the other hand, showed a decreasing immunoreactivity associated with the progression of malignant transformation (Ajioka et al., 1997; Baldus et al., 1998). However, it is distinctively expressed in mucinous compared to tubular adenocarcinomas (Blank et al., 1994; Ajioka et al., 1996; Hanski et al., 1997; Baldus et al., 1998). However, in all of these studies only selected antigens were investigated, while a systematically conducted comparative characterization of tissues derived from the same patient series was never performed.

Therefore, the aim of the present work was to provide such a comparative investigation of Lewis blood group and mucin peptide core antigens in the colorectal adenoma-carcinoma sequence. In this context, we focused on correlations of their expression with grade of dysplasia, malignant transformation, and histopathological carcinoma subtype. The quantitative relationship between oligosaccharides and mucin peptides was also determined because it may reflect the mutual relationships generated by the mucin glycoprotein biosynthesis including the complex process of glycosylation.

Materials and methods

Tissues

Specimens from 42 colorectal adenomas and 44 colorectal carcinomas were derived from the files of the Center of Pathology of the University of Cologne. They were routinely fixed in 5% phosphate-buffered formalin and embedded in paraffin. Colorectal adenomas were classified according to their histological type (tubular, tubular-villous, villous) and the grade of dysplasia, i.e. low-grade or high-grade (Meijer et al., 1995). Low-grade adenomas (n=27) were mainly polypoid-pedunculated (n=23), in part polypoid-sessile (n=1) or flat-type (n=3). Their median size was lower than 1 cm (<1 cm, 14; 1-2 cm, 10; >2 cm, 3). The high-grade adenomas were polypoid-pedunculated in 11 cases, polypoid-sessile in 3 cases and belonged to the flat-type in one case. Their size was in median greater than 2 cm (<1 cm, 2; 1-2 cm, 5, >2 cm, 8). Colorectal carcinomas were classified according to the WHO classification into (1) well or moderately differentiated tubular adenocarcinomas without relevant extracellular mucin and (2) mucinous (colloidal, gelatinous) adenocarcinomas, which are characterized by abundant extracellular mucus representing at least 50 % of the total tumor area.

Monoclonal antibodies

Monoclonal antibodies (mabs) directed against peptide and carbohydrate epitopes were purchased and diluted according to the instructions of the manufacturers as reported below. MUC1 was detected by mab HMFG2 (Coulter Immunotech, Hamburg, Germany), MUC2 and MUC5AC were visualized applying mabs NCL-MUC-2 and NCL-MUC-5AC, respectively (Novocastra, Newcastle upon Tyne, UK). Mab HMFG2 (Burchell et al., 1983) detects the DTR peptide motif on MUC1 (Taylor-Papadimitriou, 1991), i.e. a glycosylation-dependent epitope. NCL-MUC2 was generated against a synthetic peptide corresponding to a site on the MUC2 glycoprotein (Xing et al., 1992), whereas NCL-MUC5AC was obtained after immunisation against a synthetic peptide of the MUC5AC tandem repeat sequence purified by HPLC (Reis et al., 1997). Mabs CD15 (Norton and Isaacson, 1987) and Anti-Lewis Y (Adachi et al., 1988) (both Dako, Hamburg, Germany) were used in order to demonstrate $Lewis^x$ and $Lewis^y$ antigens. $Sialyl\text{-}Lewis^a$-specific mab NCL-Ca19-9 (Haglund et al., 1989) was purchased from Novocastra. $Sialyl\text{-}Lewis^x$ was detected by mab CD15s (Becton-Dickinson Pharmingen, Hamburg, Germany) (Fukushima et al., 1984).

Immunohistochemistry

Following deparaffinization of 5 µm-thick sections according to routine histological techniques, a streptavidin-biotin-peroxidase assay was performed as described earlier (Baldus et al., 1998). Shortly, after blocking with 1% H_2O_2/methanol and normal swine serum, monoclonal antibodies (or normal mouse serum as negative control) were incubated for 30 min at room temperature (RT). In the next steps, biotinylated rabbit-anti-mouse immunoglobulins (DAKO, Copenhagen, Denmark, E413, 1:300) and streptavidin-peroxidase-conjugate (DAKO P397, 1:400) were applied under the same conditions. The reaction was visualized by 200 µg/ml 3-amino-9-ethyl-carbazol (Sigma, Munich, Germany) in 50 mM sodium actetate/5% dimethyl-formamide/ 0.01% H_2O_2 (30 min, RT). Tissue sections were counterstained with haematoxylin and mounted in glycerol jelly.

If more than 5% of the neoplastic tissue contained staining products at a magnification of x400, a specimen was reported to be positively stained. The immunoreactivity was scored as indicated: 0, 0-5%; 1, >5-35%; 2, >35-65%; 3, >65-100%. Statistical analyses were performed applying the chi-square test at a

significance level of 5%. In order to investigate the relationship between the expression of carbohydrate and mucin peptide core antigens, differences of staining scores were evaluated. Therefore, for each case the difference between each mucin peptide staining score (MUC1, MUC2, MUC5AC) and each carbohydrate epitope (sialyl-Le^a, Le^x, Le^y, sialyl-Le^x) was calculated. Based on these data, the median values of the differences between each peptide and carbohydrate score were evaluated. A positive median staining score difference indicates a stronger peptide reactivity in most of the cases, whereas a negative median staining score difference reflects a higher carbohydrate expression compared to the respective mucin peptide. Furthermore, associations between peptide and carbohydrate epitopes were evaluated by the Kendall rank correlation.

Results

Colorectal adenomas

If the staining score of colorectal adenomas was correlated with the histological type, immunoreactivity of sialyl-Lewis^a, Le^y and MUC5AC showed statistically significant differences (Table 1), especially Le^y and MUC5AC exhibited a stronger binding to tubular-villous and villous adenomas compared to tubular adenomas. Especially the two villous adenomas demonstrated a considerable immunoreactivity. The pattern of reactivity of Le^y, MUC1 and MUC2 was also very strongly correlated to the grade of dysplasia (Table 2), whereas the other antigens did not exhibit such a relationship (Fig. 1a,b). The staining score of Le^y and MUC1

Table 1. Immunoreactivity of mucin-associated carbohydrate and peptide antigens according to the histological type of adenomas.

HISTOLOGICAL TYPE	TUBULAR				TUBULAR-VILLOUS				VILLOUS				P
Staining score	0	1	2	3	0	1	2	3	0	1	2	3	
Sialyl-Le^a	0	4	5	10	0	2	6	13	1	0	0	1	0.0012
Le^x	3	11	1	4	3	6	5	7	1	1	0	0	NS
Sialyl-Le^x	1	8	2	8	2	3	8	8	0	0	2	0	NS
Le^y	13	3	1	2	5	3	8	5	0	0	1	1	0.0438
MUC1	13	5	1	0	6	12	3	0	2	0	0	0	NS
MUC2	2	2	12	3	3	11	4	3	0	1	1	0	NS
MUC5AC	8	9	1	1	5	13	2	1	0	0	2	0	0.0086

Table 2. Immunoreactivity of mucin-associated carbohydrate and peptide antigens according to the grade of dysplasia of adenomas.

HISTOLOGICAL TYPE	LOW-GRADE				HIGH-GRADE				p
Staining score	0	1	2	3	0	1	2	3	
Sialyl-Le^a	1	4	7	15	0	2	4	9	NS
Le^x	5	15	2	5	2	3	4	6	NS
Sialyl-Le^x	1	9	6	11	2	2	6	5	NS
Le^y	15	5	3	4	3	1	7	4	0.0221
MUC1	17	9	1	0	4	8	3	0	0.0454
MUC2	2	4	17	4	3	10	0	2	0.0003
MUC5AC	9	14	3	1	4	8	2	1	NS

Table 3. Immunoreactivity of mucin-associated carbohydrate and peptide antigens according to the histological type of carcinomas.

HISTOLOGICAL TYPE	TUBULAR				MUCINOUS				P
Staining score	0	1	2	3	0	1	2	3	
Sialyl-Le^a	5	5	5	20	0	1	1	7	NS
Le^x	8	10	8	9	0	1	4	4	NS
Sialyl-Le^x	5	3	9	18	0	3	1	5	NS
Le^y	1	4	7	23	0	1	1	7	NS
MUC1	11	14	6	4	3	6	0	0	NS
MUC2	13	18	2	2	0	3	1	5	0.0016
MUC5AC	23	7	3	2	5	2	1	1	NS

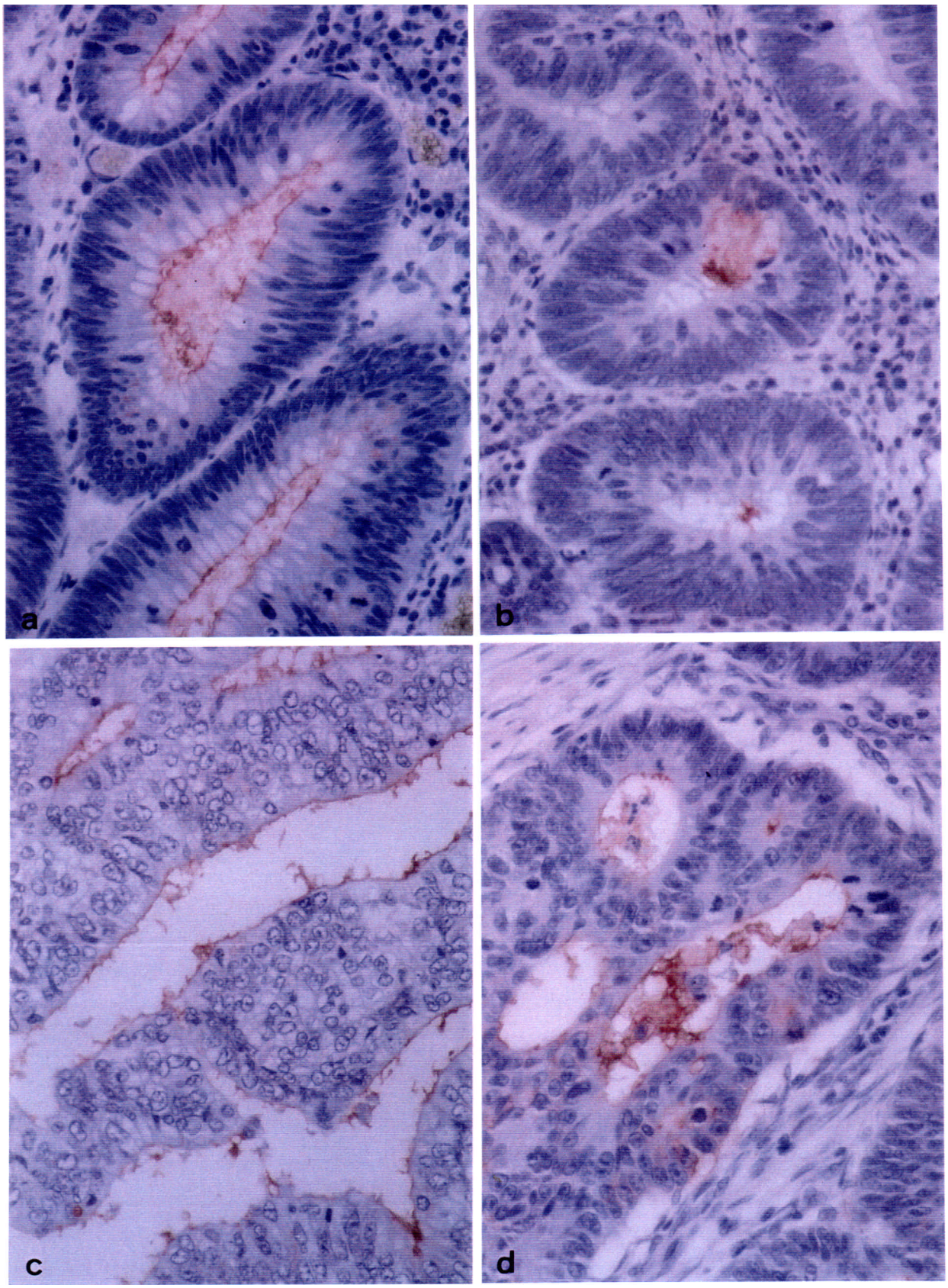

Fig. 1. The figures show representative patterns of reactivity: an adenoma with low-grade dysplasia strongly expressing sialyl-Lewis^a antigen **(a)** and an adenoma with high-grade dysplasia exhibiting Le^y antigen **(b)**. Moderately differentiated tubular adenocarcinomas showed a distinct immunoreactivity of sialyl-Lewis^x **(c)** as well as MUC1 **(d)**. x 580

antigens showed a significant increase with the development of high-grade dysplasia. MUC2, on the other hand, exhibited an inverse behaviour reflected by a decrease of reactivity accompanying the transition from low-grade to high-grade dysplasia. The staining pattern revealed additional characteristics. Whereas the other mabs bound prominently to luminal membranes or secretions, MUC2 showed a distinctively expressed cytoplasmic or supranuclear reaction. Obviously, the MUC2 decrease accompanied a reduction of mucus-secreting cells. Accordingly, MUC5AC was mainly observed as a secreted mucin.

Colorectal carcinomas

When the main histological groups (well or moderately differentiated vs. mucinous) of colorectal carcinomas were compared with regard to the staining of carbohydrate- and peptide-specific antibodies, all mabs directed against Lewis blood group antigens (Fig. 1c) were strongly reactive with both of them (Table 3). MUC1 (Fig. 1d) exhibited a tendency towards a stronger immunoreactivity in tubular compared to mucinous carcinomas. However, it was statistically non-significant. MUC2, on the other hand, showed a statistically significant inverse pattern characterized by a strong staining of mucinous, and a faint reactivity of tubular adenocarcinomas. MUC5AC did not reveal any relevant differences. With regard to the staining pattern, carbohydrate antigens and MUC1 exhibited a prominent, but not exclusive staining of luminal membranes and intratubular secretions, whereas MUC2 strongly reacted with extracellular mucus products.

Relation between the immunoreactivity of peptide and carbohydrate antigens

When the medians of staining scores differences (carbohydrate staining scores subtracted from the mucin peptide staining scores in each case) in adenomas were compared (Table 4), only MUC2 showed a stronger general immunoreactivity than Le^x and Le^y, as reflected by a positive median staining score difference. The expression of MUC1 and Le^y, MUC2 and sialyl-Le^x, MUC5AC and Le^x as well as Le^y were similar (median score difference 0). In all the other combinations, carbohydrate antigens were mostly stronger reactive (negative median staining scores differences). In high-grade adenomas, the median of all staining score differences was negative, indicating a stronger immunoreactivity of all Lewis blood group compared to all mucin peptide core antigens (Table 4). Tubular adenocarcinomas (Table 5) generally exhibited the same relationship, with the exception of MUC1 and Le^x, which showed a comparable reactivity (median staining score difference 0). In contrast, mucinous adenocarcinomas were characterized by an increase of MUC2 compared to the carbohydrate antigens. This is reflected by a a median staining score difference of 0 (Table 5), whereas Lewis blood group antigens were stronger reactive than MUC1 and MUC5AC (median staining score difference -2), as represented by Table 5. Additionally, Kendall rank correlation analyses between peptide and carbohydrate immunoreactivity scores were performed. However, they resulted in very low tau values. Significant positive or negative correlations were not obtained.

Discussion

The present study was performed in order to evaluate the relationships between the expression of (carbohydrate) Lewis blood group antigens and mucin peptide cores in colorectal adenomas. Up to now, studies focusing on a selected panel of these antigens revealed in part conflicting data based on tissues from different

Table 4. Immunoreactivity score differences of mucin peptide and carbohydrate epitopes in colorectal adenomas.

SCORE DIFFERENCE	LOW-GRADE ADENOMAS		HIGH-GRADE ADENOMAS	
	(Range)	Median	(Range)	Median
MUC1 - sialyl-Le^a	(-3; 1)	-2	(-3; 1)	-2
MUC1 – Le^x	(-3; 1)	-1	(-3; 2)	-1
MUC1 – Le^y	(-3; 1)	0	(-2; 2)	-1
MUC1 – sialyl-Le^x	(-3; 1)	-2	(-3; 2)	-1
MUC2 – sialyl-Le^a	(-3; 2)	-1	(-3; 0)	-1
MUC2 – Le^x	(-3; 3)	1	(-3; 1)	-1
MUC2 – Le^y	(-3; 3)	1	(-2; 1)	-1
MUC2 – sialyl-Le^x	(-3; 2)	0	(-3; 1)	-1
MUC5AC – sialyl-Le^a	(-3; 2)	-2	(-3; 1)	-2
MUC5AC – Le^x	(-3; 2)	0	(-2; 2)	-1
MUC5AC – Le^y	(-3; 3)	0	(-3; 1)	-1
MUC5AC – sialyl-Le^x	(-3; 0)	-1	(-3; 1)	-1

Table 5. Immunoreactivity score differences of mucin peptide and carbohydrate epitopes in colorectal carcinomas.

SCORE DIFFERENCE	TUBULAR CARCINOMAS		MUCINOUS CARCINOMAS	
	(Range)	Median	(Range)	Median
MUC1 - sialyl-Le^a	(-3; 3)	-1	(-3; 1)	-2
MUC1 – Le^x	(-3; 3)	0	(-2; 0)	-2
MUC1 – Le^y	(-3; 3)	-1	(-3; 1)	-2
MUC1 – sialyl-Le^x	(-3; 3)	-1	(-3; 0)	-2
MUC2 – sialyl-Le^a	(-3; 1)	-2	(-2; 3)	0
MUC2 – Le^x	(-3; 3)	-1	(-2; 2)	0
MUC2 – Le^y	(-3; 1)	-2	(-2; 3)	0
MUC2 – sialyl-Le^x	(-3; 1)	-2	(-2; 2)	0
MUC5AC – sialyl-Le^a	(-3; 2)	-2	(-3; 2)	-2
MUC5AC – Le^x	(-3; 2)	-1	(-3; 1)	-2
MUC5AC – Le^y	(-3; 1)	-3	(-3; 2)	-2
MUC5AC – sialyl-Le^x	(-3; 1)	-2	(-3; 2)	-2

patient collectives including the application of various histological classifications and gradings. From the group of Lewis blood group antigens, only Le^y showed a correlation with a tubular-villous or villous histological phenotype and the development of high-grade dysplasia in colorectal adenomas according to our data. This result extends former reports of a correlation between the expression of Le^y and its trifucosylated variant with severe dysplasia in colorectal adenomas (Kim et al., 1986; Waldock et al., 1989). Furthermore, it resembles some α3-polyfucosylated Le type 2 chain antigens (Yuan et al., 1987) and a α3/4-monofucosylated polylactosaminoglycan epitope (Baldus et al., 1995). On the other hand, the α3-monofucosylated Le^x antigen and its α2-3-sialylated variant sialyl-Le^x did not exhibit a significant correlation with the development of high-grade dysplasia in the present investigation. The latter result is contradictory to a previous study, which reported a significant association of the immunoreactivity of sialyl-Le^x specific mab AM-3 (Hanisch et al., 1992) with severe dysplasia in the colorectal adenoma-carcinoma sequence (Hanski et al., 1990). Staining of two Le^x-specific mabs correlated with polyp size in another study, but only one of them bound more strongly to adenomas with severe dysplasia (Yuan et al., 1987). Sialyl-Le^a, corresponding to Ca19-9 antigen, did not show an association with the grade of dysplasia according to our data. The same observation was made by other investigators (Tucci et al., 1999), whereas a significant correlation with severe dysplasia had also been reported previously (Afdhal et al., 1987).

On analysis of mucin peptide antigens, MUC1 showed a significantly stronger expression in adenomas with severe dysplasia. Similar observations have been previously reported (Cao et al., 1997; Baldus et al., 1998). In contrast, MUC2 was significantly more reactive in low-grade adenomas, in concordance with two other investigations (Ajioka et al., 1997; Baldus et al., 1998). In previous analyses, RNA message levels of MUC1 were significantly enhanced in adenomas with increasing villous histology, size and dysplasia, whereas MUC2 scores were only higher in adenomas of greater villous histology and size (Ho et al., 1996). MUC5AC was reported to exhibit a decrease in immunohistochemical staining reaction and/or mRNA level accompanying increase in dysplasia (Buisine et al., 1996; Bartman et al., 1999). However, in the tissue specimens investigated in our study its immunoreactivity did not show any significant difference.

So far, possible differences between the common well or moderately differentiated tubular colorectal carcinomas and mucinous adenocarcinomas have not been thoroughly investigated. We failed to observe relevant differences, since the great majority of carcinomas contained the Lewis antigens without respect to the histological subtype. The mucin peptide cores, on the other hand, exhibited distinct differences: MUC2 score was significantly higher in mucinous compared to tubular carcinomas, as repeatedly reported (Ho et al., 1993; Blank et al., 1994; Ajioka et al., 1996; Hanski et al., 1997; Baldus et al., 1998). In contrast, MUC1 was more strongly reactive in tubular cancers, confirming earlier observations (Baldus et al., 1998). MUC5AC was previously not extensively investigated with respect to the histological carcinoma subtype, but it did not show significant differences according to our data.

Besides the analysis of single mucin peptide or carbohydrate epitopes, the analysis of possible relationships between them seems to be of special interest. Such an attempt was made in a former study (Ajioka et al., 1996). The authors observed a positive correlation between MUC1 and Le^y, and a negative correlation between MUC2 and Le^y (applying Kendall rank coefficients). Comparing our data, we could not establish such significant differences, but by calculating differences between the staining scores of mucin peptides and carbohydrate epitopes similar conclusions could be deduced: Le^y binding was enhanced, compared to MUC1 immunoreactivity, during the progression of the adenoma-carcinoma sequence, whereas sialyl-Le^a, Le^x and sialyl-Le^x did not show such a tendency. In contrast, MUC2 score diminished compared to the score of all Lewis antigens. However, all these results have to be interpreted cautiously because of the influences of glycosylation on the binding properties of mucin peptide-specific antibodies. HMFG2, for example, detects a glycosylation-dependent part of the MUC1 peptide core, i.e. the DTR sequence, and MUC2-specific mabs recognize non-glycosylated peptide sequences. However, biochemical as well as immunochemical data regarding the glycosylation of normal or neoplastic colorectal mucin are very scarce up to now. A presence of sialyl-Le^x on MUC1 and MUC2 could be described (Hanski et al., 1995), and MUC1 glycoproteins bearing Thomsen-Friedenreich epitopes (i.e. Galß1-3GalNAc core epitopes) were demonstrated by sandwich ELISA assays (Baldus et al., 1998).

All these data suggest that MUC1 peptide immunoreactivity is enhanced during the course of the colorectal adenoma-carcinoma sequence leading to the development of tubular adenocarcinomas, whereas MUC2 shows an inverse behavior. However, it remains speculative, if MUC peptide cores receive a carrier peptide-specific glycosylation, especially with regard to peripheral carbohydrate antigens (like the Lewis blood group antigens) substituting the type 1 or type 2 backbone chains. In this context, further bio- and immunochemical investigations are warranted in order to clarify these relations in tumor tissues.

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